LOCALIZATION OF FRANCISELLA PATHOGENICITY ISLAND-ENCODED SECRETED PROTEINS AND THEIR SECRETION SYSTEM

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Abstract

Intracellular pathogens have evolved virulence genes that allow them to exploit host cells for their life cycles, and virulence genes are commonly located in pathogenicity islands, such as the *Francisella* pathogenicity island of *Francisella tularensis*. The *Francisella* pathogenicity island is linked to virulence, intracellular growth, and a type VI secretion system. Since the *Francisella* pathogenicity island encodes a secretion system, I hypothesize that *Francisella* pathogenicity island encoded proteins are secreted during infection of host cells. The molecular mechanisms involved in the pathogenesis of this bacterium are not well understood and there are no readily available tools for studying these mechanisms. Therefore, I developed expression plasmids of all *Francisella* pathogenicity island encoded proteins as C-terminal and N-terminal epitope FLAG-tagged proteins. The *Francisella* pathogenicity island encoded proteins expressed from these plasmids successfully restored the intramacrophage growth phenotype in mutants of their respective genes that were deficient for intramacrophage growth. Immuno-fluorescence microscopy experiments of cells infected with bacteria containing the expression plasmids showed some of the *Francisella* pathogenicity island encoded proteins were secreted. To test if protein localization is dependent on the type VI secretion system, localization observed in wild type was compared to the localization of *Francisella* pathogenicity island encoded proteins in a *pdpB* mutant, a gene that is homologous to a type VI secretion system structural inner membrane protein. The localization of FLAG-tagged proteins was significantly reduced when expressed in the *pdpB* mutant compared to expression in wild type. Two of the secreted proteins, *pdpC* and *pdpE,* were tested for their roles in pathogenicity. *pdpC* was required for virulence *in vivo* but not for growth within macrophages. Plasmid expression of PdpC-FLAG and FLAG-PdpC in the *pdpC* mutant restored the virulent phenotype to that of the wild type. PdpE was not required for

intramacrophage growth or virulence in mice. These data further support the hypothesis that the *Francisella* pathogenicity island encodes a secretome that contributes to the virulence of *Francisella*.

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Chapter 1: Introduction of *Francisella tularensis* Host Cell Interactions

1.1 Bacteria-Host Cell Interactions

Bacteria are found throughout the biosphere, existing in environments ranging from the Arctic tundra to acid hot springs where thermophiles thrive. Due to the widespread distribution and nature of bacteria, humans have continual contact and interactions with them. The majority of bacteria exist in neutral or symbiotic relations with us, although some bacteria are pathogenic. However, pathogenic bacteria evolved distinct interactions with their hosts that allow them to elude host defenses. Host defense mechanisms are mediated by the immune system. The immune response includes highly specialized cells such as macrophages, dendritic cells, and neutrophils; these specialized cells are capable of engulfing microbial organism by the process of phagocytosis [1]. Phagocytic cells have specialized pattern recognition receptors that bind surface-exposed macromolecules displayed by microorganisms [2].

1.2 Phagocytosis and Phagosomal Maturation

Phagocytized microbes are first internalized and trapped within a phagosome, where they are subjected to microbicidal features designed to destroy invading microbes [3]. For microbicidal destruction, the phagosome undergoes a maturation process by gaining and losing membrane proteins that control fusion and fission events, including the delivery of vesicles containing anti-microbial enzymes to the phagosome [4]. These surface proteins of the phagosome are used to distinguish between three stages of phagosomal maturation: early phagosomal, late phagosomal, and the phagolysosome [4]. The defining membrane protein of the early phagosomal membrane is GTPase Rab5, which is responsible for the initial vesicular fusion

events to the phagosome [5]. Rab5 recruits a phosphatidylinositol 3-phosphate (PI3P) producing complex known as p150-hVPS34 to the phagosomal membrane. PI3P anchors the vesicle-controlling protein EEA1 to the phagosomes cytosolic membrane [6]. A limited number of membrane-spanning ATPases translocate H^+ into the phagosome, hindering microbial growth by hydrolytic enzymes and generating reactive oxygen species (ROS). The early stage of phagosomal maturation is mildly acidic (6.1-6.5 pH) [7].

As the phagosome matures from the early to the late phagosomal stage the number of H^+ ATPases associated with its membrane increases, making the interior of the phagosome more acidic (pH 5.5-6.0) [4]. Also, vesicular fusion events lead to the accumulation of proteases and lysosomal associated membrane proteins (LAMP) to the phagosome during the late phagosomal stage [4]. GTPase Rab7 is the distinguishing protein of the late phagosomal stage, which controls trafficking of the late phagosome [5]. Rab7 aids the fusion of lysosomes with the late phagosome, resulting in the complete maturation of a phagolysosome.

The phagolysosome is a degradative organelle that has an increased concentration of cathepsin, a protease, and lacks the mannose-6-phosphate receptor [8]. The phagolysosome is highly acidic (pH 4.5), and generates ROS. ROSs are produced by the transfer of electrons to oxygen by the membrane-associated NADPH oxidase complex of the phagosome [3]. Microbicidal nitric oxide diffuses through the membrane into the phagosome and is converted to a range of reactive nitrogen species (RNS) that target bacterial components inside the phagosome. Specifically, RNS and ROS target thiols, nucleic acids, and lipids resulting in bacterial protein inactivation [9]. In addition to RNS and ROS, phagosomes contain antimicrobial enzymes such as proteases and hydrolases; these enzymes interfere with microbial functions and destroy bacterial structures [10]. Also the phagosomes contain lactoferrins that

further inhibit bacterial proliferation by sequestering iron, which is required for bacterial growth [11]. Phagocytic cells are able to eliminate the majority of bacteria encountered through this arsenal of antimicrobial factors.

The degradation of microbes within professional phagocytic cells like macrophages, leads to antigen presentation on the surface of the phagocytic cells [2]. Antigen presentation leads to activation of the adaptive immune response with T-cells, B-cells, and specific antibody production [2]. This demonstrates the interaction of the innate and adaptive immune response in clearance of infection [12].

1.2.1 Avoiding Phagosomal Destruction

Some pathogenic bacteria have evolved mechanisms to avoid phagocytosis. Obligate and facultative intracellular pathogens utilize a variety of mechanisms for surviving within host cells, and facultative intracellular pathogens have evolved complex pathways to regulate their virulence factors [13]. Bacteria occupy different niches including several different host cell types such as phagocytic cells, and within host cells there are different compartments that bacteria occupy such as a membrane bound vacuoles or the cytoplasm [13]. Bacterial defenses against phagocytic degradation occur at every step of phagocytosis, and fall into three different strategies: i). escaping the phagosome, ii). resistance to the phagosome environment, and iii). manipulation of phagosome maturation [14, 15, 16].

Listeria monocytogenes is a facultative intracellular pathogen and the causative agent of listeriosis, an acute intestinal-tract infection normally contracted after ingestion of contaminated foods [14, 17]. When *L. monocytogenes* is taken up in phagocytic cells, pore-forming molecules such as listeriolysin O (LLO) and phospholipase CB become activated by acidification of the

phagosome (pH 5.5**)** [18]. LLO is an enzyme that inserts into the phagosomal membrane by binding cholesterol and forming pores, leading to vacuolar disruption [18, 19, 20]. Once the bacterium is in the cytoplasm, *L. monocytogenes* can replicate and polymerize actin filaments to move around the host cell or enter neighboring cells [21]. This bacterium triggers an autophagic response later during infection; the autophagic response normally results in degradation of invading bacteria, however, *Listeria* inhibits the maturation of the autophagosome with LLO and resides in non-degradative double membrane vacuoles [22].

Another manipulation of phagosome maturation strategy utilized by facultative intracellular pathogens involves the inhibition of lysosome fusion with the phagosome. *Salmonella* species are able to survive within the phagosome by remodeling the phagosome and inhibiting phagosome maturation. *Salmonella* species induce expression of proteins when the bacterium is engulfed in phagocytes; these proteins block and prevent fusion of the phagosome with the lysosome [23]. Upon acidification of the phagosome, *Salmonella* down regulates *Salmonella* pathogenicity island 1 and up regulates *Salmonella* pathogenicity island 2, expressing a type 3 secretion system and secreted effector proteins that alter host cell behavior [23]. Within macrophages, *Salmonella* can induce host cell death at different times during infection through these various mechanisms [24, 25].

1.3 Pathogenicity Islands

Pathogenic, intracellular bacteria and their hosts are continuously co-evolving. Genes that encode virulence factors are often found within an organism's pathogenicity island (PI) [26]. PIs exist in several pathogenic bacteria and are absent in non-pathogenic species [26]. Not all pathogenic bacteria posses a PI, such as *Listeria* [27]. PIs are mobile genetic elements of linked

genes that have been horizontally acquired, which suggest a selective advantage for organisms in ecological fitness or resistance in the context of its pathogenicity [28]. Common characteristics of PIs include the presence of virulence genes that are flanked by insertion-like elements of direct repeat sequences and tRNA genes, and PIs are associated with G+C content that is different compared to the core genome of the organism [26, 29]. These genetic elements encode virulence factors that include adhesions, toxins, invasion proteins, and secretion systems [26, 29]. *Salmonella typhimurium* has multiple PIs, the genes of which encode proteins that are secreted within the host cell and the secretion apparatus itself.

1.4 Secretion Systems

Many virulence factors are secreted proteins. A number of proteins such as toxins and effectors are destined to enter the host cell. Secretion systems vary in complexity and are usually recognized by a set of core components that comprise the structure of the secretion devices. A limited number of different secretion systems have been described. These secretion systems have different mechanisms of transporting proteins from within the bacteria to the extracellular matrix. A major pathway for transporting proteins across the plasma membrane or integrating proteins into the plasma membrane is the secretion dependent or Sec-dependent pathway, which is also referred to as the general secretion pathway and is highly conserved in both prokaryotes and eukaryotes [30, 31]. Another pathway utilized by bacteria is the twin arginine translocation pathway (TAT), where two consecutive arginines act as a signal sequence for secretion via this pathway [31]. TAT is able to transport folded proteins across the membrane [31].

The type I secretion system (T1SS) is also known as the ATP binding cassette (ABC) protein secretion, which exports bacterial toxins [30]. T1SS is Sec-independent and crosses the

inner and outer bacterial membranes in a continuous manner [31]. That is, proteins are secreted from bacterial cytoplasm directly to the extracellular matrix. T1SS is dependent on a C-terminal secretion signal [30]. *Escherichia coli* has a T1SS that secretes HlyA, which enters the extracellular matrix, binds to a host cell, and forms a pore in the host cell membrane [30, 31].

Proteins secreted via the type II secretion system (T2SS) are Sec-dependent [30, 31]. Proteins are first transported across the plasma membrane via Sec or Tat, and then the T2SS transports proteins from the periplasmic space across the outer membrane to the extracellular matrix. Proteins that are secreted through the T2SS possess an N-terminal signal sequence that targets proteins for secretion [30]. *Vibrio cholerae* has a T2SS that secretes the cholera toxin [30].

The type III secretion system (T3SS) is present in many pathogenic bacteria including *Salmonella enterica*, *Shigella flexneri, E. coli*, and others [30, 32]. The T3SS acts as a molecular syringe, injecting proteins directly into the host cell and is, therefore, considered to be contact dependent with the host cell [30, 32]. Effector proteins are translocated continuously through both bacterial membranes in a Sec-independent manner, and the T3SS is able to transport proteins through up to three membranes at once, two bacterial and one host [30].

Type IV secretion systems (T4SS) transfer both proteins and single stranded DNA-protein complexes [30]. Transfer of DNA from one bacterium to another is a method of horizontal gene transfer amongst bacteria. Transport of material through the T4SS can be either cell contact-dependent, like the molecular syringe of the T3SS secreting proteins through three membranes, or independent of cell contact, transporting the protein to the extracellular matrix [30].

The type V secretion system (T5SS) is Sec-dependent; proteins are transported from cytoplasm to periplasmic space via Sec [30]. However, T5SS is unique in that proteins recently transported via Sec insert themselves into the outer membrane. These proteins, referred to as autotransporters, create their own channel in which they transport themselves to the extracellular matrix [30].

The type VI secretion system (T6SS) has been identified in *Vibrio cholerae* and other bacteria; however, the T6SS is not yet well characterized. The core cluster believed to be associated with the T6SS was initially named IAHP for IcmF-associated homologous proteins because they contain an IcmF-like protein (intracellular multiplication F) [33]. Although IcmF and DotU are core requirements of the T6SS, they are also associated with the T4SS. IcmF and DotU are localized to the inner membrane [33]. IcmF-like proteins contain several transmembrane domains and a Walker-A nucleotide-binding motif, and the C-terminus of DotU contains a transmembrane segment [34]. IcmF is required for intramacrophage growth of *L. pneumophila* [34, 35]. *icmF and dotU* mutants have similar growth phenotypes, and the stability of the IcmF-DotU paradigm requires the presence of both genes, suggesting these two gene products work together [33, 34].

Mycobacterium species have a type VII secretion system (T7SS) [36]. This secretion system is specialized in its ability to transport proteins through the characteristic waxy hydrophobic cell wall of *Mycobacterium* into the extracellular matrix.

Recently the extracellular nucleation precipitation (ENP) pathway has been described as a type VIII secretion system [37]. The ENP pathway of *E. coli* is required for secretion and assembly of curli, which are adhesive fibers on the surface of some bacterial pathogens that interact with host cells and biofilm formation. Data on the ENP pathway are conflicting; some

data suggests the ENP pathway is a channel through the bacterial membranes, while other data suggests a chaperone assists in translocation of the outer membrane [37].

1.5 *Francisella tularensis*

Francisella tularensis was first isolated and named *Bacterium tularensis* by Dr. Edward Francis, who studied this bacterium shortly after an outbreak in 1911 of a plague-like disease affecting ground squirrels in Tulare County, California [38, 39]. *Francisella tularensis* is a gram-negative, aerobic, coccobacillus, capsule forming, facultative intracellular bacterium that causes disease in humans and other animals. There are varying degrees of virulence among the four subspecies of *F. tularensis: F. tularensis* subspecies *tularensis, F. tularensis* subspecies *mediasiatica, F. tularensis* subspecies *holarctica,* and *F. tularensis* subspecies *novicida* [40, 41]. *F. tularensis* subspecies *tularensis* is also known as Type A and is the most virulent subspecies. Type A is found in North America. *F. tularensis* subspecies *holarctica,* or Type B, is found throughout the northern hemisphere. *F. tularensis* subspecies *mediasiatica,* is found in Central Asia. Whether *F. tularensis* subspecies *novicida* should be classified as a subspecies or its own species is currently in debate [42, 43]. This subspecies is relatively avirulent in people; however, *F. novicida* is genetically similar to *F. tularensis*. *F. novicida* produces a disease in mice in a similar manner to the way *F. tularensis* produces disease in humans [43]. These reasons explain why *F. novicida* is an acceptable model for studying the mechanisms of bacterial host cell interactions of *F. tularensis.*

1.5.1 *Francisella* Disease and Treatment

F. tularensis is an extremely infectious organism that is the causative agent of tularemia [38, 39]. In 2005 the Center for Disease Control reported 165 cases of tularemia in the United States. The occurrence of tularemia is believed to be higher than reported because tularemia is treated symptomatically and not confirmed by laboratories. The subspecies *tularensis* and *holarctica*, which are both of clinical significance due to their ability to establish infections in otherwise healthy humans, have varying severities depending on the route of transmission. An infection with *tularensis* can be lethal, while *holarctica* results in a similar yet less severe infection [33]. *F. novicida* and *F. philomiragia* are capable of producing disease within infected immunocompromised individuals [38, 39]. The subspecies *mediasiatica* has yet to be documented causing infections in humans, although it does infect wildlife and can to their death as can *holarctica* [39].

Tularemia infection manifests itself approximately three to five days after transmission of as few as 10 organisms [40, 44]. This disease can be difficult to diagnose since symptoms parallel several other bacterial infections, such as general flu-like symptoms, malaise, delirium, vomiting and nausea, and fever and chill cycles [40, 45]. Disease involves dissemination of the organism to multiple organ systems. Tularemia is subdivided into different forms depending on the site of entry as different entries lead to different manifestations: ulceroglandular, oculoglandular, gastrointestinal, and pneumonic [40, 46].

Transmissions via arthropod vectors or skin lesions are the most common infection routes for tularemia, and lead to ulceroglandular tularemia [40]. This disease is presented by inflammation and lesion at the site of infection, which develops into an ulcer or papule [40, 47]. As the ulcer heals, nearby lymph nodes become swollen [44, 48]. Inoculation into the eye is rare

and results in oculoglandular tularemia [48]. Gastrointestinal tularemia results from consumption of contaminated water or food. This form of tularemia has a wide range of severity depending on the infectious dose, and varies from persistent diarrhea to bowel ulcers to even death [49]. Transmission from inhaled aerosolized bacteria can cause a lethal respiratory form of infection if left untreated [40, 46]. This pneumonic or respiratory tularemia is the most serious form. In addition to aerosolized bacteria, this form of tularemia can also develop from the bacteria spreading from an initial site to the lungs [44]. Inhalation of *holarctica* also causes severe disease, but is seldom fatal [50].

Tularemia is treated via antibiotic therapy. *F. tularensis* synthesizes β-lactamases, therefore, penicillin and its derivatives are ineffective; also this bacteria has a natural erythromycin resistance [40, 44]. The aminoglycoside gentamicin has been used to treat tularemia, and recently tetracycline, doxycycline, and the quinolone ciprofloxacin are being used as treatments [51].

A live-vaccine strain (LVS) derived from *F. tularensis holarctica* has been developed for protection against infection with *F. tularensis tularensis* [45]. The attenuation of this strain is undefined, raising several concerns regarding the safety of this vaccine; therefore, this vaccine is only used experimentally [40]. Also, LVS still produces disease in mice, and it is used extensively as a model organism in research.

1.5.2 Epidemiology

Most cases of tularemia occur in areas where *F. tularensis* is endemic; therefore, it is considered a disease of the Northern hemisphere, affecting Asia, North American, and Northern and Central parts of Europe [38, 40]. Epidemiological cases identify the risk factors associated

with tularemia as handling infected animals or arthropod bites. Both the exact infectious cycle and reservoir of *Francisella* are often unknown; however, *F. holarctica* infections are associated with aquatic environments [39]. This is in part due to beavers and muskrats often becoming infected and human infection occurring after consumption of contaminated water [39, 47]. Mosquitos are considered a risk factor for contracting tularemia [38]. However, it is unclear if this is from mosquito larvae hatching in watersheds becoming infected before turning into mosquitos and becoming vectors of the disease or if mosquitos feed on non-aquatic, *Francisella*-infected animals then becoming vectors. In addition to the water vectors previously discussed, there are several non-aquatic vectors that are also highly associated with disease, such as rabbits, rodents, deer, and ticks [38]. It is not known if these non-aquatic animals become infected at contaminated water sources [38, 40]. *Francisella* has been isolated from well over 200 species of animals, from mammals (including humans) to birds, fish, reptiles, and amphibians [38].

F. tularensis is considered an extremely infectious bacteria due to its relatively low inoculation dose and multiple routes of entry. The severity of respiratory tularemia in particular, has lead to its classification as a category A agent by the United States Center for Disease Control and Prevention [40, 52]. *F. tularensis* has been part of biowarfare programs of the United States, the former USSR, and Japan [52]. Interestingly, outbreaks of the disease occur in localized foci, even though the organism is ubiquitous in the Northern hemisphere. Outbreaks of tularemia in humans coincide with fatal *F. tularensis* disease in animal populations [40]. Such an event occurred on Martha's Vineyard where hares from the Western States were imported [39, 53]. Similarly, hares were also imported to Spain, and contributed to the spreading disease in Southwestern Europe [54].

Traditionally farmers and hunters were at risk for contracting tularemia [55]. Changes in farming practices and declines in hunting in the Rocky Mountain Region, may have contributed to the decrease in human tularemia, where it was once endemic [39]. The rareness of disease, yet severity of manifestations, and the intracellular life style of *Francisella* is unique among microbes and leaves much to be discovered.

1.6 *Francisella* Intracellular Life Cycle

Francisella lacks classical virulence genes such as those encoding for a T3SS or toxins, yet it has an exceptional ability to parasitize immune cells [56]. Mutagenesis studies of *Francisella* genes have identified a number of genetic loci that are important for intracellular survival of *Francisella* [57, 58]. These mutants are defective for intracellular growth and unable to cause disease, which emphasizes the importance of intracellular replication to this pathogen [59, 60]. However, most genes identified by random mutagenesis have not been complemented, and therefore, their role in intracellular growth cannot be unequivocally confirmed. In some cases, roles of homologous proteins in other organisms have been investigated. Also, the functions of the proteins encoded by these genes have not been determined.

F. tularensis invades and replicates in a variety of different cells; one vital target cell is the macrophage. *F. tularensis* has a complex intracellular life as a facultative intracellular bacterium. Uptake of bacteria is dependent on complement factors. Entry into macrophages involves the formation of asymmetric loop-like pseudopods, which encircle and engulf the organism [61]. Internalized bacteria reside in a phagosome, a membrane-bound compartment in the cell termed the *Francisella*-containing phagosome [62, 63, 64]. The structure of the phagosome has also been identified as unusual because of its dense fibrillar coat [65]. *F.*

tularensis escapes from the phagosome by degrading the membrane within 30 to 90 minutes of uptake, where it enters the cytosol and is capable of massive replication [16, 66, 67]. This ability to escape from the phagosome into the cytoplasm of the host cell has been linked to the *Francisella* pathogenicity island encoded protein intracellular growth locus C (IglC) [68, 69]. Acid phosphatases are required for both phagosomal escape and cytoplasmic replication, which eventually leads to cytopathogenicity [70, 71]. Phagosome maturation ceases during the late endosomal stages when the compartment starts acidifying. Acidification of the phagosome triggers bacteria to avoid degradation, either by inhibiting fusion of the phagosome-lysosome or by escaping from the phagosome [65].

The *Francisella*-containing phagosome initially displays the early endosomal marker EEA-1 before maturing to the late endosomal stage displaying the surface proteins: LAMP-1, LAMP-2, and CD-63 [64, 65]. However, late endosomal stage phagosomes are expected to be acidic, and data regarding the acidification of the *Francisella*-containing phagosome is contradictory [65, 72, 73]. One study showed transient acidification occurring 20min after uptake followed by a gradual decrease in acidification at 40 and 60min [63]. This suggests the *Francisella*-containing phagosome acquires transient acidification through ATPase pumps prior to phagosomal escape [65, 73]. Since inhibition of ATPase activity delays phagosomal escape of bacteria, acidification may be required for expression virulence factors required for bacterial escape [65, 73]. The observed decrease in acidity of the *Francisella*-containing phagosome may be an effect of membrane disruption allowing acidic contents to diffuse and equilibrate with the cell cytosol [65]. The late endosomal phagosome with *Francisella* does not contain cathepsin D, a degradative enzyme that is associated with the phagolysosome fusion stage [64, 65, 69]. This suggests *Francisella* is able to delay maturation of the phagosome by degrading the phagosomal

membrane [64]. Degradation of the phagosomal membrane leads to escape of *Francisella* into the host cytosol although the exact time is dependent up on the bacterial strain and cell type studied [16, 66, 73]. *Francisella* is able to delay activation of the inflammasome after phagosomal escape; inflammasome mediated cell death is activated when bacterial enter into the cytosol of macrophages [74, 75, 76]. The molecular mechanism of inflammasome inhibition during intracellular survival of *Francisella* remains to be deciphered.

Francisella replication within host cytosol occurs approximately between 5 and 20 hours post uptake resulting in a large number of bacteria in the cytosol of infected cells [16, 66]. In the cytosol during intracellular replication, *Francisella* up-regulates genes required for nutrient acquisition and several novel genes encoding intracellular survival factors [77]. After bacterial growth 20 to 24 hours post uptake, *Francisella subspecies* (*F. tularensis, F. novicida*, and *F. holarctica*) re-enter double membranous vacuoles that contain the autophagic probe MDC (monodansylcadaverine) and the autophagic protein LC3 [66, 78]. At this point during infection these vacuoles are acidified and possess lysosomal features including LAMP- 1 and cathepsin D with surviving bacteria [66, 69]. The significance of autophagosome formation to *Francisella* is unknown; however, it is speculated the autophagic response is important to *Francisella* [79]. The intracellular lifecycle of *Francisella* eventually leads to cytopathogenicity and apoptosis in infected cells [66, 71].

Progress in the field of *Francisella* cell biology has been rapid in the last decade, yet many aspects of *Francisella* intracellular life remain poorly understood. The intracellular life cycle of *Francisella* is unique and combines intracellular survival strategies of other pathogens such as phagosomal escape (*Listeria*) and modulating host cell functions (*Salmonella*). There is currently a lack of information regarding the specific virulence mechanisms in the intracellular

life style of *Francisella.* Further work describing *Francisella* intracellular survival mechanisms will facilitate the identification of novel *Francisella* virulence factors.

1.7 The *Francisella* Pathogenicity Island

Genomes of the four *F. tularensis* subspecies are sequenced. *Francisella* species contain a genetic element within their chromosome termed the *Francisella* pathogenicity island (FPI). This region is approximately 30 kilo-bases with an average G+C content significantly lower than the core chromosome of *Francisella* [60]. Two of the genes within this island, *pdpA* and *pdpE, have an* average G+C content of 26.6%; similar G+C contents to this region are found in a low G+C Gram-positive bacteria or *Plasmodium* [80]. *F. tularensis* subspecies *tularensis* and *holarctica* have a duplicated FPI, which suggests this region was once mobile [60]. The *F. novicida* and *F. philamiragia* chromosomes only harbor one copy of the FPI, and it is speculated that the additional copy of the FPI contributes to the virulence associated with *F. tularensis* and *F. holarctica* [80]. There are two putative operons that form the FPI; the *pdp* (pathogenicity determinant protein) operon consists of 12 genes spanning *pdpA* thru *pdpE,* and the *igl* (intracellular growth locus) operon on the opposite coding strand, which includes *anmk, pdpD* and *iglABCD.*

FPI genes are transcriptionally regulated. MglA is a global regulator of the FPI genes [69]. FPI genes are induced when *Francisella* is in a low-iron environment. *Francisella's* growth cycle, specifically in the late exponential phase to early stationary phases, also plays a role in up regulating virulence genes. Within host cells, the FPI is up regulated [69]. The FPI in *F. tularensis* subspecies is associated with the pathogenicity of the organism and mutations in the FPI alter intracellular growth and decrease virulence.

A number of mutagenesis studies as well as transcriptional profiling have identified genes required for intracellular growth of *Francisella;* the FPI deletion mutants of *iglABCDEFHIJ, pdpAB, dotU,* and *vgrG* are defective for intramacrophage growth [57, 58, 77, 81, 82, 83, 84]. As a regulator of the FPI, MglA is also required for intercellular growth [85]. Deletion mutations of both *iglC* genes of *F. holarctica* LVS cause a replication defect in macrophages [71]. The gene, *iglC* is highly up regulated in *Francisella* during intracellular replication [71, 82, 83]. *iglC* mutants are unable to disrupt the phagosome and enter into the cytoplasm; therefore, they are unable to replicate intracellularly, and unable to induce apoptosis in the host cell [67, 71]. A reduction of intracellular replication is seen in *Francisella* strains lacking a functional gene encoding the FPI protein IglD [67]. When compared to the wild type, *iglD* mutants reside in the late endosomal-like phagosomes before a delayed escape to the host cytoplasm. In the cytoplasm the *iglD* mutant is unable to replicate intracellularly as observed in the wild type strain [67]. Therefore, intracellular replication and phagosomal escape are two separate virulence attributes [67].

Phagosomal escape is required for *Francisella* pathogenesis [68, 69]. The FPI genes *iglC, iglD* and *pdpA* are required for phagosomal escape and are potential virulence factors of *Francisella* [72, 73, 81]. Phagosomal maturation and escape kinetics of *iglC* and *iglD* mutants have been investigated in both LVS and *F. novicida,* whereas a *pdpA* mutant has only been studied in *F. novicida*; there are subtle differences in intracellular trafficking of *iglC* and *iglD* mutants between LVS and *F. novicida.* In LVS, *iglC* and *iglD* are not required for stalling phagosome maturation but are required to escape the noxious, confined compartment [68, 72, 81]. In contrast, *F. novicida iglC* is required for phagosomal escape [69, 73, 81]. Mutants of both LVS and *F. novicida* are capable of limited replication in macrophages, which is still 100 logs

less than replicating wild type bacteria, once again stressing the importance of these genes to the intracellular life of *Francisella* [68, 69, 72]. Replication of LVS *iglC* and *iglD* mutant strains occurs in vacuoles containing LAMP-1. Similarly, a *pdpA* of *F. novicida* is required for full replication in LAMP-1 positive phagosomes [72, 81].

Enzymes and pore-forming proteins are involved in phagosomal escape of a number of pathogens, and acid phosphatases that catalyze hydrolysis of phosphomonoesters under acidic conditions are involved in intracellular survival of several bacterial species [86]. *F. tularensis* contains several acid phosphatases [70, 78, 86]. An *acpA* mutant has delayed phagosomal escape; whereas a quadruple mutant of *acpABC* and *hap* genes that encode for phosphatases renders it completely defective for phagosomal escape up to 24 h post uptake [78, 86]. This suggests a role of acid phosphatases in phagosomal escape of *Francisella.* Rather than directly acting on the phagosomal membrane, AcpA could act indirectly by activating other proteins required for phagosomal escape [70].

A number of genetic screens have identified genes needed for virulence in an animal model of infection [87, 88, 89]. The overlap between genes needed for intracellular growth and virulence indicates the importance of intracellular growth to *Francisella* [88, 89, 90]. There are also genes required for virulence, which have not been identified as necessary for intracellular growth [87]. These virulence genes encode proteins of unknown function lacking known homologues. Their contribution to virulence and phagosomal escape has yet to be investigated. Specifically the given effector proteins of secretion systems of many intracellular pathogens affect virulence, but do not influence intracellular growth kinetics [91]. Mutations in other proteins such as PdpA or PdpB (pathogenicity determining protein) also reduced virulence compared to wild type [59, 84]. The FPI is correlated with the organism's virulence and

intracellular growth; however, the cellular and molecular mechanisms of pathogenesis and the specific functions of the FPI proteins are not known.

Although the FPI does not contain every core requirement of the T6SS, there is enough evidence to argue that in the absence of a trueT6SS, there is a T6S-like-system encoded within the FPI. Core requirements of the T6SS include IcmF, DotU IcmF-like proteins, IAHP (IcmF-associated homologous proteins), a putative lipoprotein, ClpV AAA^+ ATPase, Hcp (haemolysin co-regulated protein), and VgrG (valine-glycine repeat proteins) [34, 92]. The FPI-encoded protein PdpB is homologous to IcmF. IcmF contains several transmembrane domains, which anchor it as an inner membrane component of the T6SS [34, 93].

Two other FPI-encoded proteins, IglA and IglB, are homologous to IAHPs seen in *Rhizobium leguminosarum, Salmonella enterica,* and *Vibrio cholerae* [93, 94, 95]. Mutations in IglA and IglB result in bacteria unable to escape the phagosome and replicate intracellularly [57, 60, 68, 69, 93]. IglA and IglB are soluble proteins that interact with each other forming the outer tube of the secretion system [96]. PdpE and VgrG are proteins that are secreted through the T6SS [34, 92]. *Francisella* species contain VgrG and DotU like proteins encoded within the FPI [96].

1.8 Summary

In summary, *F. tularensis* is a highly infectious bacterium that is able to avoid destruction by host macrophages. Identification of the FPI and its correlation to intracellular growth, virulence, and a secretion system has raised many questions in regards to the organism's pathogenesis to the molecular mechanisms involved in intracellular growth and virulence. In order to develop effective intervention strategies against tularemia, these molecular mechanisms must be identified. My thesis research focused on localizing the protein products of the FPI

during infection of host cells, and investigating the contribution of individual FPI genes to their localization and their roles in the intracellular growth of *F. tularensis*.

I hypothesize the FPI encodes a T6SS and the secreted proteins. To test this hypothesis, I developed the following aims. (i.) Determine the localization of FPI proteins during infection of macrophages. This specific aim will address the hypothesis that FPI-encoded proteins comprise a secretion system and secreted effector proteins. First I will develop a complete FPI expression system to track full-length, biologically active FPI proteins. Then I will use microscopy as well as cell biological and biochemical methods to determine which FPI proteins are components of the secretion system and which are secreted proteins. (ii.) Determine if PdpC is secreted during infection. This aim will address the hypothesis that PdpC is a T6SS secreted protein. First I will construct bacteria that have epitope tagged forms of the FPI encoded proteins integrated into their chromosome. Lastly I will assess the dependence PdpC localization has with the T6SS. To explore the relation of PdpC with the T6SS, I will use a *pdpB* deletion mutant to assess PdpC localization. (iii.) Assess the role of *pdpC* in virulence. This specific aim will address the hypothesis that *pdpC* is a secreted effector protein required for virulence. Virulence between wild type, *pdpC* deletion mutant, and *pdpC* restored *pdpC* deletion mutants of *Francisella* will be investigated for the role of PdpC virulence in fertilized chicken eggs.

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Chapter 2: Development of Genetic Tools for Studying the FPI[1]

2.1 Abstract

Francisella tularensis is the causative agent of the zoonotic disease tularemia. *F. tularensis* can survive and replicate within host macrophages. The molecular mechanisms involved in the pathogenesis of this bacterium are not well understood. The *Francisella* pathogenicity island (FPI) is linked to virulence, intracellular growth, and a type VI-secretion system. This study describes the development of expression plasmids for all FPI-encoded proteins with C-terminal and N-terminal epitope tags. To confirm these plasmids express their respective epitope FLAG-tagged proteins Western blots were used. All 18 of the C-terminal epitope tagged FPI-encoded proteins are expressed at their predicted molecular weights. As for the N-terminal epitope tagged FPI-encoded proteins, 17 are expressed at their predicted molecular weights. The FPI proteins expressed from these plasmids successfully restore the intramacrophage growth phenotype in mutants of the respective genes that are deficient for intramacrophage growth. These genetic tools lead to the full-length expression of biologically functional epitope tagged proteins, providing new tools for studying the molecular mechanisms of pathogenicity in *F. tularensis*.

[1] Hare RF and Hueffer K (2014) Development of Genetic Tools for Studying the FPI is prepared for submission to PLOS ONE.

2.2 Introduction

Francisella tularensis is gram-negative, coccobacillus, facultative intracellular bacteria that causes tularemia. Tularemia is a disease affecting over 200 different species of animals, including mammals (including humans), birds, fish, reptiles, and amphibians [1]. This pathogenic bacterium is listed as a category A select bioterrorism agent by the Center for Disease Control because of its extremely low infectious dose; as few as 10 cells cause disease in humans [2]. Additionally, there are multiple routes of transmission and entry [2, 3, 4].

In humans, tularemia typically manifests itself three to five days after exposure [5]. Bacterial infections vary from mild, flu-like symptoms to lethal if left untreated. *F. tularensis* synthesizes β-lactamases, which are enzymes that provide resistance to beta-lactam antibiotics by breaking the structure through hydrolyses. Therefore penicillin and its derivatives are ineffective in treating tularemia. Consequently tularemia is most often treated with tetracyclines and chloramphenicol or alternatively gentamicin [2, 6]. The occurrence of tularemia is both under diagnosed and reported because it is treated symptomatically. Depending on the site of entry, tularemia presents in different clinical forms, including ulceroglandular, oculoglandular, gastrointestinal, typhoidal, and pneumonic [2].

The *Francisella* pathogenicity island (FPI) (Fig. 2.1) is a conserved cluster of genes that contribute to its pathogenicity [7, 8]. Mutations in the FPI decrease the organism's virulence, ability to enter the cytoplasm, and intracellular growth, suggesting an association between pathogenesis and the FPI [8, 9, 10, 11, 12, 13]. Due to the decrease of intracellular growth seen in mutants of these genes, these genes are named *intracellular growth locus* (*igl*), and the mutations within genes that decrease virulence are named *pathogenicity determinate protein* (*pdp*). There are 10 *igl* genes and five *pdp* genes within the FPI. The other three remaining FPI

genes are *anhydro-N-acetylmuramic acid kinase* (*anmk*), *defect in organelle trafficking* (*dotU*), and *valine glycine repeat* (*vgrG*). The protein names for these genes' products are the same as their respective gene designation.

F. tularensis enters the host's macrophages by a process involving loop-like pseudopods [14]. Internalized bacteria initially reside within a membrane bound compartment in the cell termed the *Francisella*-containing phagosome [15, 16, 17]. *Francisella* evades phagosomal degradation by escaping from the phagosome and entering the cytosol, where it replicates. The intracellular replication of *Francisella* leads to cytopathogenicity and apoptosis in infected cells [18, 19]. The ability to enter into the cytosol of the host cell has been linked to the FPI-encoded protein IglC [17, 20]. IglC is highly up regulated in *Francisella* during intracellular replication [21]. *iglC* mutants are unable to disrupt the phagosome and enter into the cytoplasm; therefore, they are unable to replicate intracellularly and induce apoptosis in the host cell [17, 18, 19]. Mutations in *iglA* and *iglB* also result in bacteria unable to escape the phagosome and replicate intracellularly [7, 10, 20, 17, 22]. Much like wild type *Francisella*, *iglD* mutants reside in the phagosome through its maturation to a late endosomal-like phagosome before escaping to the host's cytosol, however, the *iglD* mutant is unable to replicate once it is in the cytosol [23]. Together, these studies suggest intracellular replication and phagosomal escape are two separate virulence attributes, encoded by different genes [17, 23].

Several FPI-encoded proteins, IglA, IglB, IglC, PdpB and DotU, constitute a type VI secretion-like system (T6SS) [13, 22, 24]. Secretion systems, such as the T6SS, involve the transport or translocation of effector molecules from the interior of a bacterial cell through its membranes to the exterior; protein secretion is an important mechanism for bacteria to function in their environment for adaptation and survival. Two of the FPI-encoded proteins, IglA and

IglB, are homologous to IAHP's (IcmF-associated homologous proteins) in *Rhizobium leguminosarum, Salmonella enterica,* and *Vibrio cholerae* [10, 22, 25, 26].

The molecular mechanisms that aid in the virulence of *F. tularensis* are not well understood. Although the FPI (Fig. 1) is required for virulence, intracellular growth, and encodes for a T6SS, no effector proteins have been described. Mutational analysis has demonstrated the requirement for FPI genes in growth and virulence, but the molecular role remains uncharacterized. Progress in understanding the molecular mechanisms of pathogenicity have been hampered by the restriction-modification system within *Francisella* and a lack of genetic tools. *Francisella novicida* possess a strong restriction-modification system [27]. Restriction-modification systems limit the acquisition of foreign DNA by cleaving DNA that has not been modified by methylation following DNA replication [27]. The restriction barrier reduces the transformation frequency of foreign DNA by 10^6-fold [27]. Plasmids that have been used to characterize *Francisella* species contain a large green fluorescent protein (GFP) cassette, which alters or disrupts protein folding, structure, and function [28]. Using *F. novicida* as a model for *F. tularensis,* genetic tools of the complete FPI were developed to localize proteins in effort to determine which FPI proteins are part of the secretion system and which are secreted. The *Francisella* expression plasmids designed contain both C-terminus and N-terminus epitope tagged forms of all open reading frames in the FPI.

2.3 Materials and Methods

2.3.1 Bacterial Cultures

Escherichia coli D10 (Invitrogen) was grown aerobically at 37°C on Luria-Bertani (LB) media, containing 50μg/ml of ampicillin (LBA) when appropriate for selection and maintenance.

F. novicida U112 (ATTC 15482) was cultured on tryptic soy agar (TSA) or tryptic soy broth (TSB), aerobically at 37°C. When selecting for transformants, *Francisella* was selected and maintained on TSA containing 15μg/ml of kanamycin. A complete list of bacterial strains, eukaryotic cell lines, plasmids, and primers used in this study are in Table 2.1.

2.3.2 DNA Manipulations

Restriction enzyme digests, sub-cloning, cloning, and DNA electrophoresis for *E. coli* were performed using standard cloning techniques and the E-Gel® Clonewell 0.8% SYBR Safe™ (Invitrogen Carlsbad, CA. USA) gel system [29]. By cutting the *Francisella* expression plasmid groE-GFP- pFNLTP6 with BamHI, the GFP insert was removed, leaving the groE promoter and the multiple cloning site (MCS) in place [28]. Within the MCS a fragment containing *sopB* and a triple FLAG tag from the plasmid pSB2598 was inserted [30]. Next through quick-change mutagenesis, the second NcoI site in the plasmid's kanamycin resistance gene was removed without changing the coding sequence. By modifying this second NcoI site, most of the FPI genes have been inserted into plasmids using the restriction enzymes EcoRI and NcoI within the MCS; this allows for easy primer design and cloning of C-terminus triple FLAG plasmids. Primers used to construct the *Francisella* expression plasmids with the epitope tag on the C terminus of FPI are listed in Table 2.2A. After cloning all C-terminal tagged FPI genes, pKH8, IglA-FLAG, has been modified becoming the backbone for N-terminus tagged plasmids. These modifications involved removing *iglA*, leaving the triple FLAG tag down stream of the groE promoter and the Shine-Delgarno sequences of *iglA* yet upstream of the MCS. Primers were designed for N-terminus triple FLAG-tagged FPI genes to be inserted with the restriction enzymes Xma1 and Xho1. Primers used to construct the *Francisella* expression plasmids with

the epitope tag on the N terminus of FPI are listed in Table 2.2B. PCR for cloning was done using Phusion® High-Fidelity PCR (Finnzymes®). Restriction enzyme digest was performed as described in New England BioLabs® Catalog and Technical Reference (Ipswich, MA. USA). PCR products and restriction enzyme digest products were purified via Wizard SV® Gel and PCR clean up System (Promega Madison, WI, USA). Ligations using T4 DNA ligase (Fisher Scientific) were done at 16°C for 14-16h. Plasmids were recovered from *E. coli* through PureYield™ Plasmid Miniprep System (Promega Madison, WI, USA) for screening and PureYield™ Plasmid Midiprep System (Promega Madison, WI, USA) to collect a stock for transformations. Plasmids were initially screened using restriction enzymes that were used for cloning and when applicable another restriction enzyme that would cut within the specific FPI gene. Plasmids were also screened for correct gene and triple FLAG sequence using standard Taq PCR.

2.3.3 Transforming *E. coli*

After plasmids and gene inserts were ligated, they were transformed into *E. coli* D10 using electroporation. *E. coli* was grown aerobically to mid-log phase, concentrated in 10% glycerol, and frozen in aliquots for electroporation. Electroporation was performed in a BIO-RAD Gene Pulser™ II (Hercules, CA. USA). Bacteria and plasmid DNA were mixed in a 0.2cm cuvette and electroporated at 2.5Kv, 25μF, and 200Ω. Immediately following electroporation, cells were suspended in 1ml of super optimal broth (SOB) and incubated at 37C° for 1h [31]. *E. coli* transformations were then plated on LBA with antibiotics to select for successful transformants. Plasmids collected from *E. coli* were sequenced to confirm that the FPI gene sequence was not altered before being transformed into *F. novicida.* Primers used for sequencing

are listed in Table 2.2. PCR was performed on the plasmids using BigDye® (Applied BioSystems, Foster City, CA USA); settings for the PCR are as followed: initial denature 4min at 96°C then a repeated cycle of denature at 96°C for 10s followed by annealing at 50°C for 5s and an extension at 60°C for 4min; this cycle was repeated 25 times. For sequencing, DNA was purified in Sephadex spin columns prior to sequencing (General Electric Healthcare Life Sciences Pittsburgh, PA USA). Sequencing was performed at the University of Alaska Fairbanks IAB Core Lab (Fairbanks, AK USA). Sequences were analyzed with Ridom TraceEdit (Ridom® GmbH Münster, Germany).

2.3.4 Transforming *Francisella*

These newly constructed *Francisella* expression plasmids containing the FPI open reading frames (ORF) were chemically transformed into *F. novicida* strain U112. A sub-culture of bacteria was grown aerobically at 37°C, shaking at 200rpm until mid log phase or an OD_{600nm} of 0.3-0.5. Cells were pelleted at 5,000 *x G* for 5min at room temperature. Cells were suspended in *Francisella* transformation buffer or transformation medium [32] and then 400µl of cell suspension was mixed with DNA and incubated aerobically at 37°C with shaking at 90rpm for 1h. 56µl of 10% glucose and 1ml of TSB was then added per transformation and incubated overnight aerobically at 37°C with shaking at 150rpm. Cells were plated in 100µl aliquots on freshly prepared TSA containing 15µg/ml of kanamycin. Colonies were picked, isolated, and then screened by PCR, restriction enzyme digest, and Western blotting for confirmation of successful transformation.

2.3.5 SDS-PAGE and Western Blotting

Transformants were screened by Western blots to ensure the plasmids expressed tagged proteins. Standard SDS-PAGE and Western blot techniques were performed. Whole cell lysates of both *E. coli* and U112 containing each of the epitope tagged plasmids were screened for protein expression. Broth cultures were grown over night (16h) to late log / early stationary phases, their OD_{600nm} was adjusted to 1.0 and diluted 1:2 in SDS loading buffer, containing 250mM TrisHCl pH 6.8, 10% SDS, 30% glycerol, 5% betamercaptoethanol, and 0.02% bromophenol blue. Samples were boiled for 10min, cooled to room temperature, and loaded into a gel of appropriate acrylamide percentage for the size of the predicted molecular weight of the epitope tagged proteins. Gels were placed in SDS running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) and the gel wells were loaded with samples. Proteins were separated at 130V, 200mA, for 90min. Proteins were then transferred to Immobilon-P membrane (Millipore Billerica, MA, USA) soaked in transfer buffer (20% methanol, 2.5mM Tris, 19.2mM glycine, pH 8.3) transfer of proteins was facilitated by 22V, 400mA, for 75min. After proteins were transferred, the membrane was blocked to reduce nonspecific binding. Blocking included incubation of the membrane in 5% non-fat dry milk (NFDM) in Tris-Buffered Saline and Tween® 20 (Fisher BioReagents® Fair Lawn, NJ US) solution (TBST) containing 1mM Tris, 15mM NaCl, 2mM KCl, and 0.1% Tween® 20 (Fisher BioReagents® Fair Lawn, NJ US) for 1h. To detect FLAG-tagged proteins, the membrane was incubated for 1h at room temperature in a 1/5000 dilution of monoclonal M2 mouse anti-FLAG® antibodies (Sigma Aldrich® Saint Louis, MO USA) in 5% NFDM in TBST. Membranes were rinsed three times with TBST for 5min before adding a 1/5000 dilution of Peroxidase-Goat Anti-Mouse (Zymed® Laboratories Invitrogen immundetection San Franciso, CA, USA) in 5% NFDM in TBST. The membrane was

incubated with secondary antibodies for 1h and was followed by three more rinses in TBST. To visualize protein bands, SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific Rockford, IL. USA) was incubated on the membrane for 1min at room temperature just before exposing and developing film.

2.3.6 Macrophage Growth Assay

J774A.1 mouse macrophage like cells (ATCC ® TIB-67™) were seeded in 24-well cell culture plates at 1.4 x 10^5 cells/well for 24h in complete Dulbecco's Modified Eagle Medium (cDMEM) (GIBCO® Invitrogen Grand Island, NY USA) containing 10% newborn calf serum (NCS). Cells were infected with *F. novicida* strains at a multiplicity of infection (MOI) of 1:50 (bacterium-to-macrophage). To help promote bacterial uptake after bacteria have been added, the 24-well dishes containing infected macrophages were centrifuged at 600 *x G* for 10min. Infected monolayers were incubated for 2h in DMEM to allow for phagocytosis to occur, washed five times in Hank's Phosphate Buffered Saline (HPBS) (GIBCO® Invitrogen Grand Island, NY USA). At this time, the infection is at 0h, and infected macrophages were then either lysed at this time zero or incubated at 37°C in 5% CO_2 for 24 and 48h. To determine bacterial replication, infected macrophages were lysed in 0.1% dexoxycholate in HPBS at 0, 24, and 48h post infection. The lysates were serially diluted in HPBS and plated on TSA and incubated at 37°C for 24 or 48h. The colony forming units (cfu) were enumerated, and used to plot growth curves. To perform statistical analysis, the fold replication at 48h was first determined (cfu 48 h/ cfu 0 h), and then the log of the 48h fold replication was used in a two-way ANOVA with XLSTAT to compare the means of each group in Tukey multiple comparisons ($\alpha = 0.05$).

2.4 Results and Discussion

2.4.1 Development of the *Francisella* Expression Plasmids

The biochemical and molecular mechanisms of FPI proteins within host cells are not well understood. The *Francisella* pathogenicity island (FPI) (Fig. 2.1) is required for both virulence and intracellular growth, and it also encodes for a type VI secretion system (T6SS). Using *Francisella novicida* as a model for *F. tularensis,* the *Francisella* expression plasmids have been designed as genetic tools to track proteins, identify protein localization, and further understand the role and importance of these FPI genes and proteins (Fig. 2.1 & 2.2).

These *Francisella* expression plasmids were maintained in both *E. coli* and *Francisella.* Cloning was performed in *E. coli* because *E. coli* has a faster growth rate, is relatively safe, and has the ability to host foreign DNA; all of which make cloning and transformations easier in *E. coli* compared to *Francisella* (Fig. 2.2) [28]. Plasmid sequences have been screened for corrected FPI gene insert via restriction enzyme digest that removed the gene where it had been inserted and cut within the gene at a specific site (Fig. 2.2 & 2.3). This confirms each of the correct FPI genes were inserted in the plasmid, however, this does not guarantee if the sequence of the FPI gene has been altered. To confirm the gene sequence has not been altered, plasmids have been sequenced from the groE promoter through the FPI gene and the triple FLAG tag (Fig. 2.2). PCR, digesting the plasmid with restriction enzymes, and sequencing all revealed that each *Francisella* expression plasmid contained the correct gene, and FPI gene sequences within the expression plasmid have not been altered.

2.4.2 C-terminus FLAG-tagged FPI Protein Expression in *E. coli*

Expression of FLAG-tagged proteins from the *Francisella* expression plasmid were assessed via Western blotting. The Western blot of FPI C-terminal FLAG-tagged proteins in *E. coli* confirms all of the C-terminal FLAG-tagged expression plasmids express their respective C-terminal FLAG-tagged FPI protein (Fig. 2.4). There are multiple bands of FLAG-tagged protein apart from the predicted weight of the FPI protein seen with PdpD-FLAG (Fig. 2.4A), IglB-FLAG, IglD-FLAG, IglI-FLAG (Fig. 2.4B), IglC-FLAG and VgrG-FLAG (Fig. 2.4C). Several proteins, IglB-FLAG, IglD-FLAG, IglI-FLAG, DotU-FLAG, IglC-FLAG, and VgrG-FLAG, are highly expressed and have an entire lane of FLAG proteins instead of a band at the predicted weight (Fig. 2.4). Expression of IglA-FLAG and IglG-FLAG is low (Figure 2.4C), but detectable following a longer exposure time (Figure 2.4C & D). The additionally labeled bands and over expression of *Francisella* proteins within *E. coli* are not surprising results; these FPI proteins are not native to *E. coli* and they maybe degraded or aggregate within *E. coli.* The C-terminal tagged *Francisella* expression plasmids express their respective FPI protein with a FLAG tag; N-terminal tagged *Francisella* expression plasmids also expressed their respective FPI proteins (data not shown).

2.4.3 FLAG-tagged FPI Protein Expression in *F. novicida*

All of the C-tag and N-tag *Francisella* expression plasmids have been transformed into *Francisella novicida* U112R [27]. *F. novicida* 4xU112R contains four mutations in the restriction modification system allowing *Francisella* to more readily accept the *Francisella* expression plasmids [27]. Once the *F. novicida* strain U112R contains the plasmids, plasmids are collected and then transformed into *Francisella novicida* U112 wild type and other U112 FPI-

mutants listed in Table 2.1. Plasmids are transformed into the wild type for two reasons. First, the plasmids can be maintained by *Francisella*, which increases the efficiency of transforming the plasmids into mutants for future studies, as plasmid replicated by *Francisella* will be methylated via the restriction modification system [27]. Secondly, to minimize potentially confounding effects from mutations. U112 transformants have been confirmed by PCR and restriction enzyme digest. PCR for the FPI gene and the FLAG tag confirms that all of the plasmids contained their respective FPI gene. Restriction enzyme digests also confirms gene have been properly inserted in the plasmid.

Next, expression of both C-terminal and N-terminal epitope tagged FPI proteins from *F. novicida* U112 have been confirmed by Western blotting to detect the FLAG tag. Western blots show 18 of the C-tagged and 17 of the N-tagged FPI proteins are expressed in *F. novicida* wild type (Fig. 2.5). Each FPI-encoded protein is also expressed at their predicted molecular weights, thus the C-terminal and N-terminal tags do not interrupt FPI protein expression (Fig. 2.5), with the exception of FLAG-PdpE. IglG-FLAG expression is lower than the other FPI proteins and was not visible here (Fig. 2.5C); expression of IglG-FLAG has been confirmed with longer exposure times causing over exposure with the other proteins (data not shown). To compare expression between different proteins from different blots, a comparison blot consisting of a protein from each of the size ranges shows a pattern in the level of expression observed in the different size ranges of FPI-encoded proteins. Smaller proteins (14-20kDa) have darker and more intense than the bands from the larger proteins (+100kDa) (Fig. 2.5D).

2.4.4 Intramacrophage Growth Complementation

Since the full-length expression of both C-terminal and N-terminal FLAG-tagged FPI proteins have been confirmed, the biological functionality of proteins are assessed. Since several FPI genes are needed for intracellular growth, the C-FLAG and N-FLAG *Francisella* expression plasmids are tested on their ability to complement respective knock out mutant strains. As previously described, the FPI deletion mutants of *iglABCDEFHIJ, pdpAB, dotU,* and *vgrG* are defective for intramacrophage growth [22, 28, 33, 34] (Fig. 2.6). Expression of C-terminal and N-terminal tagged FPI proteins, IglABCDEFHIJ, PdpAB, DotU, and VgrG, in FPI mutants increased growth rates, indicating that *Francisella* expression plasmids complement their mutants. Genetic complementation of each deletion mutant with the C-FLAG and N-FLAG-tagged *Francisella* expression plasmids restored intramacrophage growth, and the growth of complemented mutants were significantly higher compared to their parental mutant (Fig. 2.6) ($p < 0.05$). Expression of the tagged proteins does not always completely restore growth to that of the wild type (Fig. 2.6). Growth of 22 of 26 complements were equivalent to that of the wild type ($p > 0.05$). However, growth of the complements, FLAG-IglB, IglC-FLAG IglJ-FLAG, FLAG-IglJ, are significantly lower compared to wild type and their respective mutant ($p < 0.05$). Together, these data indicate that most of the plasmids lead to the expression of biologically functional proteins.

The *Francisella* expression plasmids are the first tools described for *Francisella* that have epitope tags for both termini for the entire set of FPI proteins. Some secreted proteins possess a secretion signal on either the N- or C-terminus [35]. Consequently, adding amino acids that tag the protein for localization may block the secretion signal, disrupting the protein's localization and function. It is unlikely that both the N- and C- termini of the proteins are required for

localization. Therefore, the *Francisella* expression plasmids are developed to contain epitope FLAG-tags at both the N- and C- termini. Moreover, the triple FLAG tag is short, and so it is unlikely to alter protein folding and function. Triple repeating sequences increase avidity of FLAG monoclonal antibodies and reduce background in Western blotting, immuno-fluorescence microscopy, and many other commercially available biochemical products specific for localizing the FLAG tag sequence.

In conclusion this study describes the development of genetic tools to assess and elucidate the function of the complete set of FPI-encoded proteins. These genetic tools include plasmids that contain an entire set of both C-terminus and N-terminus epitope triple FLAG-tagged FPI genes that express FPI proteins. Western blotting of bacterial lysates reveals expression of 35 full-length epitope tagged FPI proteins. The *Francisella* expression plasmid expresses full-length functional proteins that restore the intramacrophage growth phenotype in respective mutants. Therefore, the *Francisella* expression plasmids are genetically viable tools that can be used to further understand the intracellular life cycle of *F. tularensis* and elucidate potential intervention strategies. These plasmids can be used in a variety of microscopic and biochemical methods to determine the localization and secretion of FPI proteins within infected cells. Also they can be used to investigate redundant functions within the FPI, as so many of the proteins are required for intracellular growth. These plasmids can also be used to determine the components of the secretion system, and the order of which they construct the secretion system. These plasmids have already been used to determine the solubility properties of the FPI proteins [24]. Overall these plasmids will contribute to a better understanding of the molecular mechanisms involved in the intracellular life cycle of *F. tularensis*

2.5 Acknowledgments

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2.6 References

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2.7 Chapter 2 Tables and Figures Legends

2.7.1 Table 2.1 Bacterial Strains Used in This Study

Descriptions of bacterial strains used in this study: (A) Parental strains and parental strains containing parental plasmids, (B) *E. coli* DH5α with *Francisella* expression plasmid, (C) *F. novicida* U112R with *Francisella* expression plasmid, (D & E) wild type *F. novicida U112* with *Francisella* expression plasmid, and wild type *F. novicida U112* with *Francisella* expression plasmid.

2.7.2 Table 2.2 Primers Used in This Study

The description contains the FPI ORF nomenclature, which terminal the tag is fused to, direction of the primer, restriction enzyme used in cloning the respective ORF, the sequence of the primer in 5'-3' direction, underlining represents in frame codon. C-terminal FLAG *Francisella* expression plasmid primers are listed in Table 2A and N-terminal FLAG *Francisella* expression plasmid primers are listed in Table 2B.

2.7.3 Table 2.3 Plasmids Used in This Study

This table lists all the plasmids used in designing the *Francisella* expression plasmids and all of the *Francisella* expression plasmids that were generated in this study.

2.7.4 Figure 2.1 Diagram of FPI in *F. novicida*

Each arrow represents an open reading frame, whose names are shown below the arrows. Arrows are drawn approximately to scale in base pairs. *pdpA* through *pdpE* represent one operon, and the region encompassing *anmK* through *iglD* constitute a second operon within the FPI. Nomenclature is as previously described: *intracellular growth locus ABCDEFGHIJ*, (*iglABCDEFGHIJ), pathogenicity determinate protein ABCD (pdpABCDE), defect in organelle trafficking U (dotU), valine glycine repeat G* (*vgrG),* and *anhydro-N-acetylmuramic acid kinase* (*anmK*) [12].

2.7.5 Figure 2.2 Experimental Outline

This diagram shows the experimental outline of the development and validation of the *Francisella* expression plasmid as a genetic tool. Developing the plasmid includes modification of pFNLTP6 groE GFP and confirmation of the correct FPI gene sequence.

2.7.6 Figure 2.3 *Francisella* Expression Plasmids

Representative diagram of the *Francisella* expression plasmids, pKH4 containing *iglC* with a C-terminal FLAG tag and pKH46 containing *iglC* with a N-terminal FLAG tag, are shown as examples of all 36 plasmids. All of the *Francisella* expression plasmids contain a groE promoter, a multiple cloning site (MCS), triple FLAG epitope tag, antibiotic cassettes, and an origin of replication. The MCS shows restriction enzyme sites used for insertion of FPI genes. Each of the FPI genes was individually inserted where *iglC* is depicted in the diagram. Arrows represent the direction of transcription and size of gene products.

2.7.7 Figure 2.4 C-terminal FLAG-tagged Protein Expression in *E. coli*

Whole cell lysates have been analyzed for production of triple FLAG-tagged proteins by Western blotting. *E. coli* and *E. coli* expressing the respective FPI protein from the *Francisella* expression plasmid are labeled above each lane. FPI-encoded proteins have been grouped into similar sizes 156-95kDa, 67.6-30.9, and 24.6-14.5 and respectively subjected to 8% (A), 10% (B), 15% SDS-PAGE (C), and (D) a 10% gel for comparisons.

2.7.8 Figure 2.5 FPI FLAG-tagged Protein Expression in *F. novicida*

Whole cell lysates have been analyzed for production of N- terminal and C-terminal FLAG-tagged proteins by Western blotting. *F. novicida* U112 wild type and U112 expressing the respective FPI protein from the *Francisella* expression plasmid are labeled above each lane. C-terminally tagged proteins are referred to as protein-FLAG, and N-terminally tagged proteins are referred to as FLAG-protein. FPI-encoded proteins have been grouped into similar predicted sizes (A) 156-95kDa, (B) 67.6-30.9, (C) 24.6-14.5, and (D) a 10% gel for comparisons. FLAG-tagged proteins are detected with monoclonal M2 mouse anti-FLAG® antibodies, goat anti-mouse conjugated HRP, and a chemiluminescent substrate.

2.7.9 Figure 2.6 Intracellular Growth

J774 cells are infected with indicated strains and harvested at 0, 24 and 4h post infection. The amount of intracellular bacteria at each time point have been determined as described in materials and methods. The *FPI* mutant lacks all FPI genes and is defective for intracellular growth, as are each of the individual FPI gene mutants. All of the genetic complements express the cognate FPI gene as a C-terminal or an N-terminal triple FLAG-tagged version from a

plasmid vector. Graphs are the average of four independent experiments and error bars show standard deviations. Statistical significance was determined via Tukey multiple comparisons with a two-way ANOVA.

Chapter 2 Tables

Table 2.1A Bacterial Strains Used in This Study

Parental strains and parental strains containing parental plasmids	Description	Reference
E. coli DH5α	Sub cloning competent cells	Invitrogen
F. novicida U112	*Francisella novicida* prototype strain Utah 112	ATTC
F. novicida U112R 2008	U112, Δrestriction genes	[27]
JLO	*F. novicida* U112 with deletion of gene FTN1758	[12]
Δ*pdpA*	U112 Δ*pdpA*	[34]
Δ*pdpB*	U112 Δ*pdpB*	[24]
Δ*iglE*	U112 Δ*iglE*	[24]
Δ*vgrG*	U112 Δ*vgrG*	[24]
Δ*iglF*	U112 Δ*iglF*	[24]
Δ*iglG*	U112 Δ*iglF*	[24]
Δ*iglH*	U112 Δ*iglH*	[24]
Δ*dotU*	U112 Δ*dotU*	[24]
Δ*iglI*	U112 Δ*iglI*	[24]
Δ*iglJ*	U112 Δ*iglJ*	[24]
Δ*pdpC*	U112 Δ*pdpC*	[24]
Δ*pdpE*	U112 Δ*pdpE*	[24]
Δ*iglD*	U112 Δ*iglD*	[24]
Δ*iglC*	U112 Δ*iglC*	[22]
Δ*iglB*	U112 Δ*iglB*	[24]
Δ*iglA*	U112 Δ*iglA*	[24]
Δ*pdpD*	U112 Δ*pdpD*	[12]
E. coli DH5α + pFLNTP6 groE GFP	groE driving GFP	[28]
E. coli DH5α + pKH1	Removed GFP from pFLNTP6	This work
E. coli DH5α + pSB2598	SopB-FLAG	[30]
E. coli DH5α + pKH2	Remove Nco1	This work
E. coli DH5α + pKH3	SopB-FLAG	This work

Table 2.1B Bacterial Strains Used in This Study

***E. coli* DH5α with *Francisella* expression plasmid**	**Descriptions**	**Reference**
E. coli DH5α + pKH22	PdpA-FLAG	This work
E. coli DH5α + pKH40	FLAG-PdpA	This work
E. coli DH5α + pKH24	PdpB-FLAG	This work
E. coli DH5α + pKH50	FLAG-PdpB	This work
E. coli DH5α + pKH9	IglE-FLAG	This work
E. coli DH5α + pKH34	FLAG-IglE	This work
E. coli DH5α + pKH10	VgrG-FLAG	This work
E. coli DH5α + pKH35	FLAG-VgrG	This work
E. coli DH5α + pKH26	IglF-FLAG	This work
E. coli DH5α + pKH44	FLAG-IglF	This work
E. coli DH5α + pKH11	IglG-FLAG	This work
E. coli DH5α + pKH36	FLAG-IglG	This work
E. coli DH5α + pKH12	IglH-FLAG	This work
E. coli DH5α + pKH45	FLAG-IglH	This work
E. coli DH5α + pKH13	DotU-FLAG	This work
E. coli DH5α + pKH37	FLAG-DotU	This work
E. coli DH5α + pKH14	IglI-FLAG	This work
E. coli DH5α + pKH39	FLAG-IglI	This work
E. coli DH5α + pKH15	IglJ-FLAG	This work
E. coli DH5α + pKH47	FLAG-IglJ	This work
E. coli DH5α + pKH5	PdpC-FLAG	This work
E. coli DH5α + pKH41	FLAG-PdpC	This work
E. coli DH5α + pKH16	PdpE-FLAG	This work
E. coli DH5α + pKH38	FLAG-PdpE	This work
E. coli DH5α + pKH6	IglD-FLAG	This work
E. coli DH5α + pKH48	FLAG-IglD	This work
E. coli DH5α + pKH4	IglC-FLAG	This work
E. coli DH5α + pKH46	FLAG-IglC	This work
E. coli DH5α + pKH18	IglB-FLAG	This work
E. coli DH5α + pKH43	FLAG-IglB	This work
E. coli DH5α + pKH8	IglA-FLAG	This work
E. coli DH5α + pKH27	FLAG-IglA	This work
E. coli DH5α + pKH7	PdpD-FLAG	This work
E. coli DH5α + pKH42	FLAG-PdpD	This work
E. coli DH5α + pKH25	AnmK-FLAG	This work
E. coli DH5α + pKH49	FLAG-AnmK	This work

Table 2.1C Bacterial Strains Used in This Study

***F. novicida* U112R with *Francisella* expression plasmid**	**Descriptions**	**Reference**
F. novicida U112R 2008+ pKH22	PdpA-FLAG	This work
F. novicida U112R 2008+ pKH40	FLAG-PdpA	This work
F. novicida U112R 2008+ pKH24	PdpB-FLAG	This work
F. novicida U112R 2008+ pKH50	FLAG-PdpB	This work
F. novicida U112R 2008+ pKH9	IglE-FLAG	This work
F. novicida U112R 2008+ pKH34	FLAG-IglE	This work
F. novicida U112R 2008+ pKH10	VgrG-FLAG	This work
F. novicida U112R 2008+ pKH35	FLAG-VgrG	This work
F. novicida U112R 2008+ pKH26	IglF-FLAG	This work
F. novicida U112R 2008+ pKH44	FLAG-IglF	This work
F. novicida U112R 2008+ pKH11	IglG-FLAG	This work
F. novicida U112R 2008+ pKH36	FLAG-IglG	This work
F. novicida U112R 2008+ pKH12	IglH-FLAG	This work
F. novicida U112R 2008+ pKH45	FLAG-IglH	This work
F. novicida U112R 2008+ pKH13	DotU-FLAG	This work
F. novicida U112R 2008+ pKH37	FLAG-DotU	This work
F. novicida U112R 2008+ pKH14	IglI-FLAG	This work
F. novicida U112R 2008+ pKH39	FLAG-IglI	This work
F. novicida U112R 2008+ pKH15	IglJ-FLAG	This work
F. novicida U112R 2008+ pKH47	FLAG-IglJ	This work
F. novicida U112R 2008+ pKH5	PdpC-FLAG	This work
F. novicida U112R 2008+ pKH41	FLAG-PdpC	This work
F. novicida U112R 2008+ pKH16	PdpE-FLAG	This work
F. novicida U112R 2008+ pKH38	FLAG-PdpE	This work
F. novicida U112R 2008+ pKH6	IglD-FLAG	This work
F. novicida U112R 2008+ pKH48	FLAG-IglD	This work
F. novicida U112R 2008+ pKH4	IglC-FLAG	This work
F. novicida U112R 2008+ pKH46	FLAG-IglC	This work
F. novicida U112R 2008+ pKH18	IglB-FLAG	This work
F. novicida U112R 2008+ pKH43	FLAG-IglB	This work
F. novicida U112R 2008+ pKH8	IglA-FLAG	This work
F. novicida U112R 2008+ pKH27	FLAG-IglA	This work
F. novicida U112R 2008+ pKH7	PdpD-FLAG	This work
F. novicida U112R 2008+ pKH42	FLAG-PdpD	This work
F. novicida U112R 2008+ pKH25	AnmK-FLAG	This work
F. novicida U112R 2008+ pKH49	FLAG-AnmK	This work

Table 2.1D Bacterial Strains Used in This Study

***F. novicida U112* with *Francisella* expression plasmid**	**Descriptions**	**Reference**
F. novicida U112 + pKH22	PdpA-FLAG	This work
F. novicida U112 + pKH40	FLAG-PdpA	This work
F. novicida U112 + pKH24	PdpB-FLAG	This work
F. novicida U112 + pKH50	FLAG-PdpB	This work
F. novicida U112 + pKH9	IglE-FLAG	This work
F. novicida U112 + pKH34	FLAG-IglE	This work
F. novicida U112 + pKH10	VgrG-FLAG	This work
F. novicida U112 + pKH35	FLAG-VgrG	This work
F. novicida U112 + pKH26	IglF-FLAG	This work
F. novicida U112 + pKH44	FLAG-IglF	This work
F. novicida U112 + pKH11	IglG-FLAG	This work
F. novicida U112 + pKH36	FLAG-IglG	This work
F. novicida U112 + pKH12	IglH-FLAG	This work
F. novicida U112 + pKH45	FLAG-IglH	This work
F. novicida U112 + pKH13	DotU-FLAG	This work
F. novicida U112 + pKH37	FLAG-DotU	This work
F. novicida U112 + pKH14	IglI-FLAG	This work
F. novicida U112 + pKH39	FLAG-IglI	This work
F. novicida U112 + pKH15	IglJ-FLAG	This work
F. novicida U112 + pKH47	FLAG-IglJ	This work
F. novicida U112 + pKH5	PdpC-FLAG	This work
F. novicida U112 + pKH41	FLAG-PdpC	This work
F. novicida U112 + pKH16	PdpE-FLAG	This work
F. novicida U112 + pKH38	FLAG-PdpE	This work
F. novicida U112 + pKH6	IglD-FLAG	This work
F. novicida U112 + pKH48	FLAG-IglD	This work
F. novicida U112 + pKH4	IglC-FLAG	This work
F. novicida U112 + pKH46	FLAG-IglC	This work
F. novicida U112 + pKH18	IglB-FLAG	This work
F. novicida U112 + pKH43	FLAG-IglB	This work
F. novicida U112 + pKH8	IglA-FLAG	This work
F. novicida U112 + pKH27	FLAG-IglA	This work
F. novicida U112 + pKH7	PdpD-FLAG	This work
F. novicida U112 + pKH42	FLAG-PdpD	This work
F. novicida U112 + pKH25	AnmK-FLAG	This work
F. novicida U112 + pKH49	FLAG-AnmK	This work

Table 2.1E Bacterial Strains Used in This Study

FPI mutant strains with *Francisella* expression plasmid	**Descriptions**	**Reference**
Δ*pdpA* + pKH22	PdpA-FLAG	This work
Δ*pdpA* + pKH40	FLAG-PdpA	This work
Δ*pdpB* + pKH24	PdpB-FLAG	This work
Δ*pdpB* + pKH50	FLAG-PdpB	This work
Δ*iglE* + pKH9	IglE-FLAG	This work
Δ*iglE* + pKH34	FLAG-IglE	This work
Δ*vgrG* + pKH10	VgrG-FLAG	This work
Δ*vgrG* + pKH35	FLAG-VgrG	This work
Δ*iglF* + pKH26	IglF-FLAG	This work
Δ*iglF* + pKH44	FLAG-IglF	This work
Δ*iglG* + pKH11	IglG-FLAG	This work
Δ*iglG* + pKH36	FLAG-IglG	This work
Δ*iglH* + pKH12	IglH-FLAG	This work
Δ*iglH* + pKH45	FLAG-IglH	This work
Δ*dotU* + pKH13	DotU-FLAG	This work
Δ*dotU* + pKH37	FLAG-DotU	This work
Δ*iglI* + pKH14	IglI-FLAG	This work
Δ*iglI* + pKH39	FLAG-IglI	This work
Δ*iglJ* + pKH15	IglJ-FLAG	This work
Δ*iglJ* + pKH47	FLAG-IglJ	This work
Δ*pdpC* + pKH5	PdpC-FLAG	This work
Δ*pdpC* + pKH41	FLAG-PdpC	This work
Δ*pdpE* + pKH16	PdpE-FLAG	This work
Δ*pdpE* + pKH38	FLAG-PdpE	This work
Δ*iglD* + pKH6	IglD-FLAG	This work
Δ*iglD*+ pKH48	FLAG-IglD	This work
Δ*iglC* + pKH4	IglC-FLAG	This work
Δ*iglC* + pKH46	FLAG-IglC	This work
Δ*iglB* + pKH18	IglB-FLAG	This work
Δ*iglB* + pKH43	FLAG-IglB	This work
Δ*iglA* + pKH8	IglA-FLAG	This work
Δ*iglA* + pKH27	FLAG-IglA	This work
Δ*pdpD* + pKH7	PdpD-FLAG	This work
Δ*pdpD* + pKH42	FLAG-PdpD	This work

Table 2.2A Primers Used in This Study

C-terminal FLAG *Francisella* expression plasmid primers
pdpA_C_terminalFLAG_F_Nde1:ggcagCATATGctaattaagtagacaatgatagc
pdpA_C_terminalFLAG_B_Nco1: ggcagCCATGGGatttccttttgatttatat
pdpB_C_terminalFLAG_F_KpnI1: agGGTACCcaaaaggaaattaaaagtatg
pdpB_C_terminalFLAG_B_Nco1: agCCATGGGttgtacattaacttctccttg
iglE_C_terminal_F_EcoR1: aggaGAATCCggcaaaaacaaggagaagttaatg
iglE_C_terminal_B_Nco1: gacgCCATGGCatctttttctatgctgctatc
vgrG_C_terminal_F_EcoR1: gagaGAATTCgattaaggggatattcttatg
vgrG_C_terminal_B_Nco1: agagCCATGGCtccaaccattgttgctgcggaacc
iglF_C_terminal_F_Nde1: gcagCATATGcaatggttggataataatatg
iglF_C_terminal_B_Nco1: agcaCCATGGCattttccaataagcttcttgcttgc
iglG_C_terminal_F_EcoR1: agagGAATTCgaagcttattggaaaatttaaatg
iglG_C_terminal_B_Nco1: agagCCATGGCagatgtttttacatttatttg
iglH_C_terminal_F_EcoR1: agagGAATTCcttagaaggtcattatcatg
iglH_C_terminal_B_Nco1: agagCCATGGCtatagagttatttaaaacaatc
dotU_C_terminal_F_EcoR1: aggaGAATTCctatataaaggatattagaaatg
dotU_C_terminal_B_Nco1: aggaCCATGGCccagcttaataaaattag
iglI_C_terminal_F_EcoR1: cgagGAATTCgggtaagaggagatttatatg
iglI_C_terminal_B_Nco1: gaagCCATGGCtatgtcaaaaagatcttc
iglJ_C_terminal_F_EcoR1: caagGAATTCcaaatgagatagatg
iglJ_C_terminal_B_Nco1: agcgCCATGGCtaaattaaaataacc
pdpC_C_terminal_F_EcoR1: ggcgaGAATTCgataaattaaggaagtacatatg
pdpC_C_terminal_B_Nco1: ggcagCCATGGGtgacgatatttttttaaaaaagtc
pdpE_C_terminal_F_EcoR1: gaagGAATTCcttaaggatgcaaaaatatg
pdpE_C_terminal_B_Nco1: aggcCCATGGCtattatagtaattttcttttc
iglD_C_terminal_F_EcoR1: ggcagGAATTCaagatcggagttgattctaatg
iglD_C_terminal_B_Nco1: ggcgaCCATGGGagaaaaggctataaagaaatc
iglC_C_terminal_F_EcoR1: gaGAATTCaaaggagaatgattatgagtgag
iglC_C_terminal_B_Nco1: gaCCATGGGtgcagctgcaatatatcc
iglB_C_terminal_F_Nde1: gagaCATATGgtagagaggattttgttatg
iglB_C_terminal_B_Nco1: agagCCATGGCgttattatttgtacc
iglA_C_terminal_F_EcoR1: agagGAATTCgtaaaaaaaggacaataagatg
iglA_C_terminal_B_Nco1: ggaaCCATGGCcttatcatctacttg
pdpD_C_terminal_F_EcoR1: ggcgaGAATTCgtaagagtagtaagtatggatcaag
pdpD_C_terminal_B_Nco1: ggcgaCCATGGGaacccagatcattggtctatac
anmK *Cterminal*F: agagGAATTCgaatataaatattgtgtaggaatcatg
anmK *CterminalB*Nco1: aggaCCATGGCaaagaaatttatttgacc

Table 2.2B Primers Used in This Study

N-terminal FLAG *Francisella* expression plasmid primers
pdpA_N_terminal_F_Xma1: acggCCCGGGgaatagcagtaaaagatataac
pdpA_N_terminal_B_Xho1: acggCTCGAGttaatttccttttgatttatatc
pdpB_N_terminal_F_Xma1: acggCCCGGGgaaattttattaaaaatcatc
pdpB_N_terminal_B_Xho1: acggCTCGAGttattgtacattaacttctccttg
iglE_N_terminal_F_Xma1: acggCCCGGGgatacaataaattattgaaaaatc
iglE_N_terminal_B_Xho1: acggCTCGAGttaatctttttctatgctgc
vgrG_N_terminal_F_Xma1: acggCCCGGGgatcaaaagcagaccatattttc
vgrG_N_terminal_B_Not1: caggGCGGCCGCttatccaaccattgttgctgcgg
iglF_N_terminal_F_Xma1: acggCCCGGGgaaataatgatattgataaatgg
iglF_N_terminal_B_Xho1: acggCTCGAGttaaattttccaataagcttcttgc
iglG_N_terminal_F_Xma1: acggCCCGGGgattaaatattataaatgactcc
iglG_N_terminal_B_Xho1: acggCTCGAGctaagatgtttttacatttatttgtcc
iglH_N_terminal_F_Xma1: acggCCCGGGatgaaaaaagaaaagatttaag
iglH_N_terminal_B_Xho1: acggCTCGAGttatatagagttatttaaaacaatc
dotU_N_terminal_F_Xma1: acggCCCGGGgaaaagactttaaagagatag
dotU_N_terminal_B_Xho1: acggCTCGAGttaccagcttaataaaattagtaagc
iglI_N_terminal_F_Xma1: acggCCCGGGgaagtcagataatatctacac
iglI_N_terminal_B_Xho1: acggCTCGAGttatatgtcaaaaagatcttc
iglJ_N_terminal_F_Xma1: acggCCCGGGgaaagactattttgaagatctttttg
iglJ_N_terminal_B_Xho1: acggCTCGAGtcataaattaaaataacctagatatatc
pdpC_N_terminal_F_Xma1: acggCCCGGGgaaacgacaaatatgaactaaatatc
pdpC_N_terminal_B_Xho1: acggCTCGAGctatgacgatatttttttaaaaaag
pdpE_N_terminal_F_Xma1:acggCCCGGGgaagtaaaaaagtatttcaattattattaatatttg
pdpE_N_terminal_B_Xho1: acggCTCGAGttatattatagtaattttcttttc
iglD_N_terminal_F_Xma1: acggCCCGGGgatttctagaaaggatttattg
iglD_N_terminal_B_Xho1: acggCTCGAGttaagaaaaggctataaagaaatc
iglC_N_terminal_F_Xma1: acggCCCGGGgaattatgagtgagatgataacaag
iglC_N_terminal_B_Xho1: acggCTCGAGctatgcagctgcaatatatcc
iglB_N_terminal_F_Xma1: acggCCCGGGgaacaataaataaattaag
iglB_N_terminal_B_Xho1: acggCTCGAGttagttattatttgtaccg
iglA_N_terminal_F_Xma1: acggCCCGGGcaaaaaataaaatcccaaattc
iglA_N_terminal_B_Not1: caggGCGGCCGCctacttatcatctacttgttgattac
pdpD_N_terminal_F_Xma1: acggCCCGGGatcaagatatcaacgatttattatatg
pdpD_N_terminal_B_Xho1: acggCTCGAGttaaacccagatcattggtctatac
anmk_N_terminal_F_Xma1: acggCCCGGGgatctggaacatcactagatgg
anmk_N_terminal_B_Xho1: acggCTCGAGttaaaagaaatttatttgacc

Table 2.3 Plasmids Used in This Study

Plasmid	Description	Reference
pFNLTP6-gro-gfp	groE-gfp; Km^r Ap^r	[28]
pKH1	Km^r Ap^r	This study
pSB2598	sopB-FLAG	[30]
pKH2	Km^r Ap^r	This study
pKH3	gro-sopB-FLAG; Km^r Ap^r	This study
pKH22	gro-pdpA-FLAG; Km^r Ap^r	This study
pKH24	gro-pdpB-FLAG; Km^r Ap^r	This study
pKH9	gro-iglE-FLAG; Km^r Ap^r	This study
pKH10	gro-vgrG-FLAG; Km^r Ap^r	This study
pKH26	gro-iglF-FLAG; Km^r Ap^r	This study
pKH11	gro-iglG-FLAG; Km^r Ap^r	This study
pKH12	gro-iglH-FLAG; Km^r Ap^r	This study
pKH13	gro-dotU-FLAG; Km^r Ap^r	This study
pKH14	gro-iglI-FLAG; Km^r Ap^r	This study
pKH15	gro-iglJ-FLAG; Km^r Ap^r	This study
pKH5	gro-pdpC-FLAG; Km^r Ap^r	This study
pKH16	gro-pdpE-FLAG; Km^r Ap^r	This study
pKH6	gro-iglD-FLAG; Km^r Ap^r	This study
pKH4	gro-iglC-FLAG; Km^r Ap^r	This study
pKH18	gro-iglB-FLAG; Km^r Ap^r	This study
pKH8	gro-iglA-FLAG; Km^r Ap^r	This study
pKH7	gro-pdpD-FLAG; Km^r Ap^r	This study
pKH25	gro-anmK-FLAG; Km^r Ap^r	This study
pKH40	gro-FLAG-pdpA; Km^r Ap^r	This study
pKH50	gro-FLAG-pdpB; Km^r Ap^r	This study
pKH34	gro-FLAG-iglE; Km^r Ap^r	This study
pKH35	gro-FLAG-vgrG; Km^r Ap^r	This study
pKH44	gro-FLAG-iglF; Km^r Ap^r	This study
pKH36	gro-FLAG-iglG; Km^r Ap^r	This study
pKH45	gro-3FLAG-iglH; Km^r Ap^r	This study
pKH37	gro-FLAG-dotU; Km^r Ap^r	This study
pKH39	gro-FLAG-iglI; Km^r Ap^r	This study
pKH47	gro-FLAG-iglJ; Km^r Ap^r	This study
pKH41	gro-FLAG-pdpC; Km^r Ap^r	This study
pKH38	gro-LAG-pdpE; Km^r Ap^r	This study
pKH48	gro-FLAG-iglD; Km^r Ap^r	This study
pKH46	gro-FLAG-iglC; Km^r Ap^r	This study
pKH43	gro-FLAG-iglB; Km^r Ap^r	This study
pKH27	gro-FLAG-iglA; Km^r Ap^r	This study
pKH42	gro-FLAG-pdpD; Km^r Ap^r	This study
pKH49	gro-FLAG-anmK; Km^r Ap^r	This study

Chapter 2 Figures

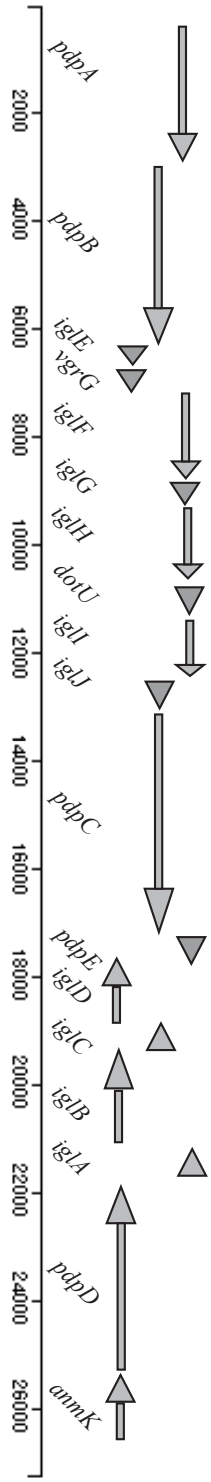

Figure 2.1 Diagram of FPI in *F. novicida*

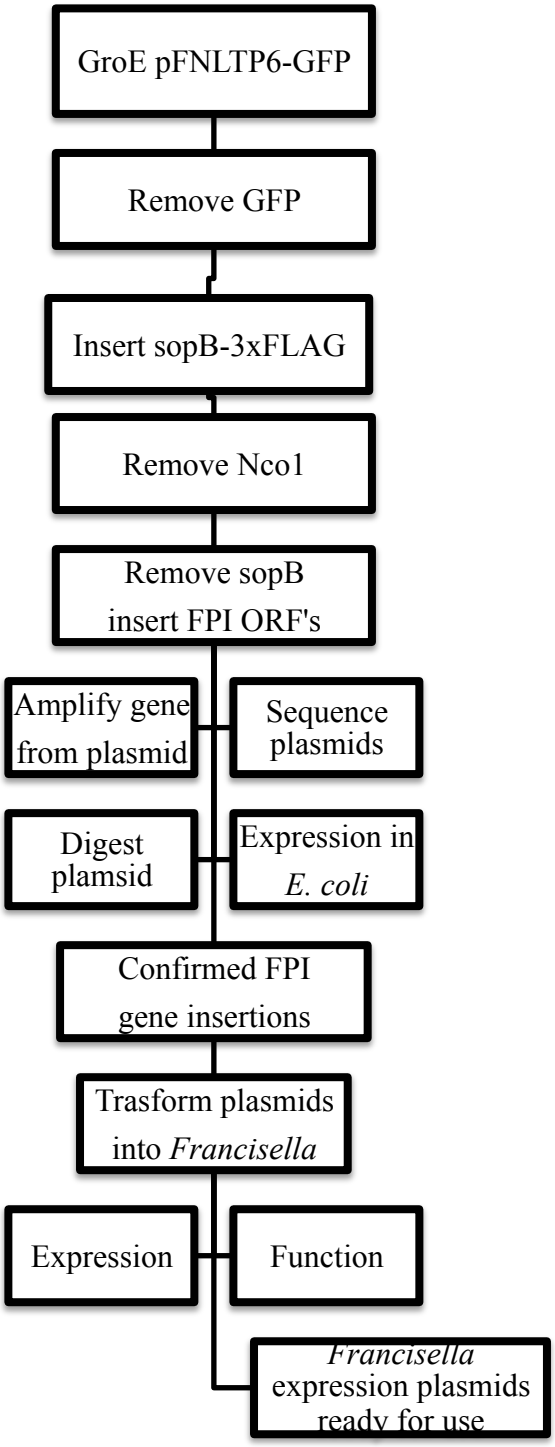

Figure 2.2 Experimental Outline

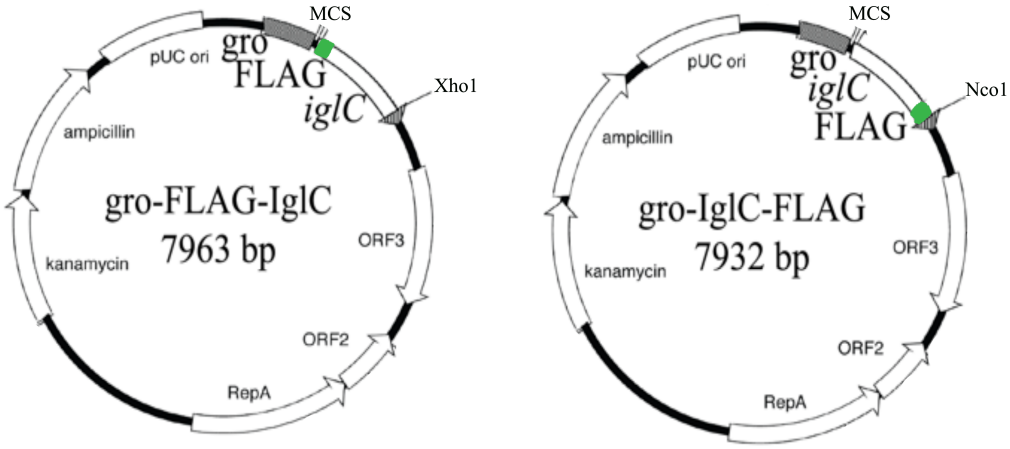

Figure 2.3 ***Francisella*** **Expression Plasmids**

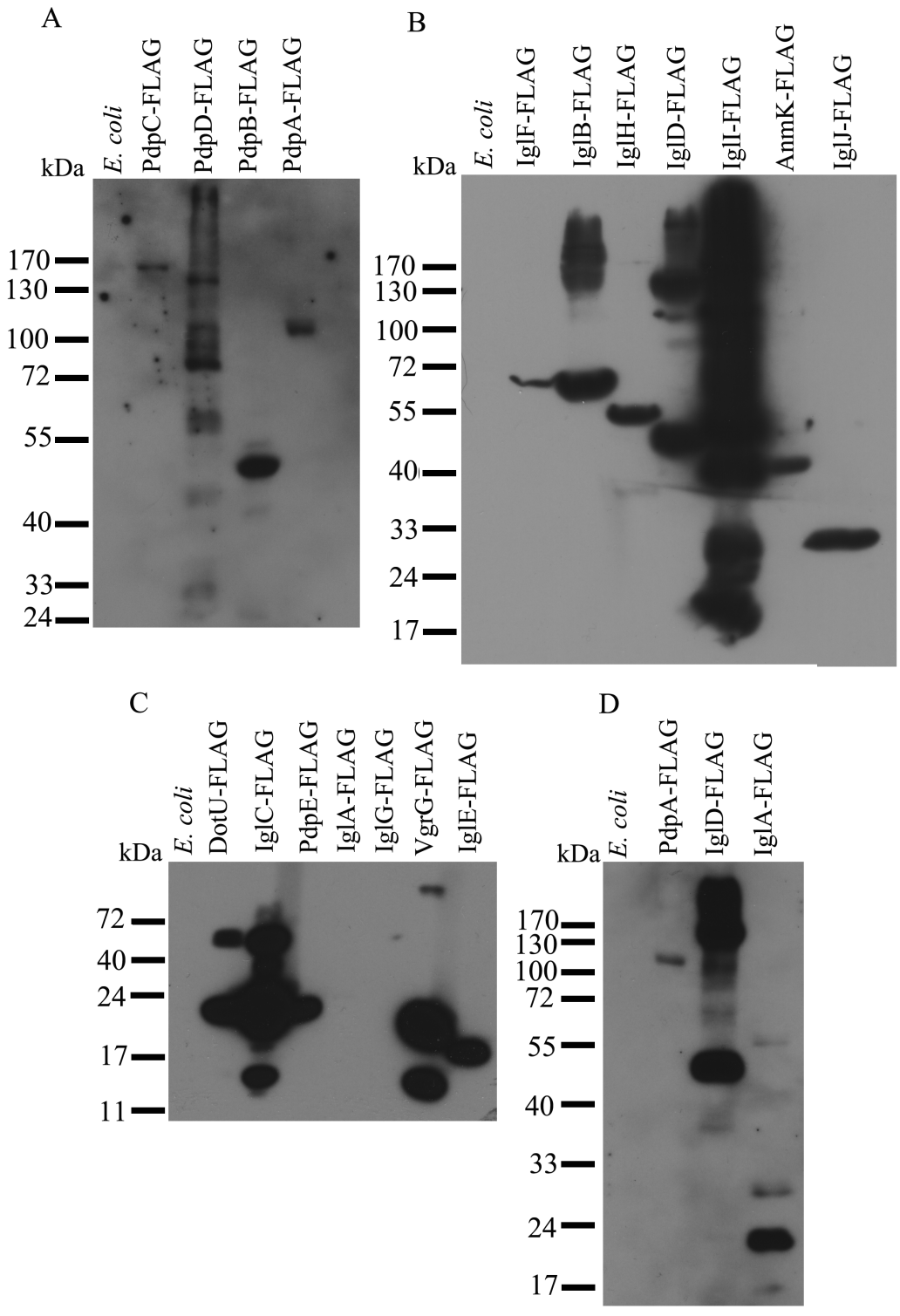

Figure 2.4 C-terminal FLAG-tagged Protein Expression in *E. coli*

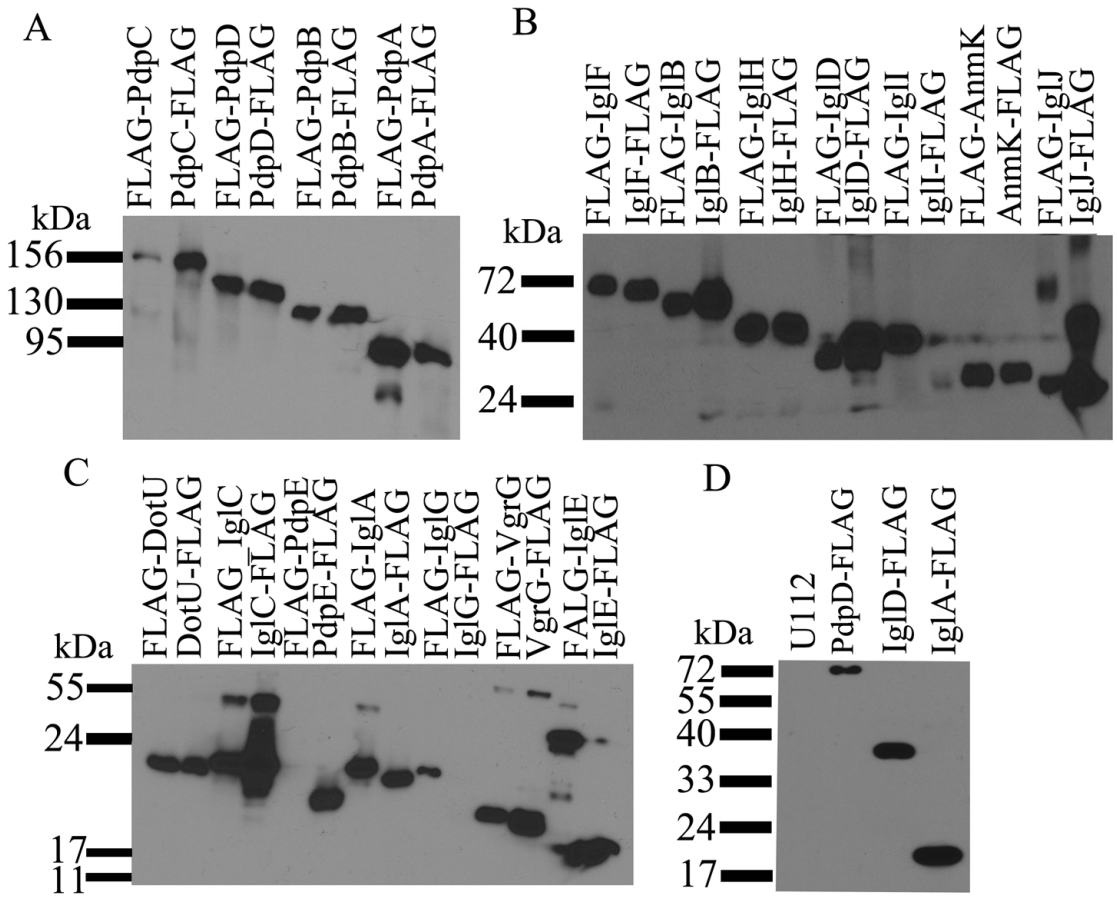

Figure 2.5 FPI FLAG-tagged Protein Expression in *F. novicida*

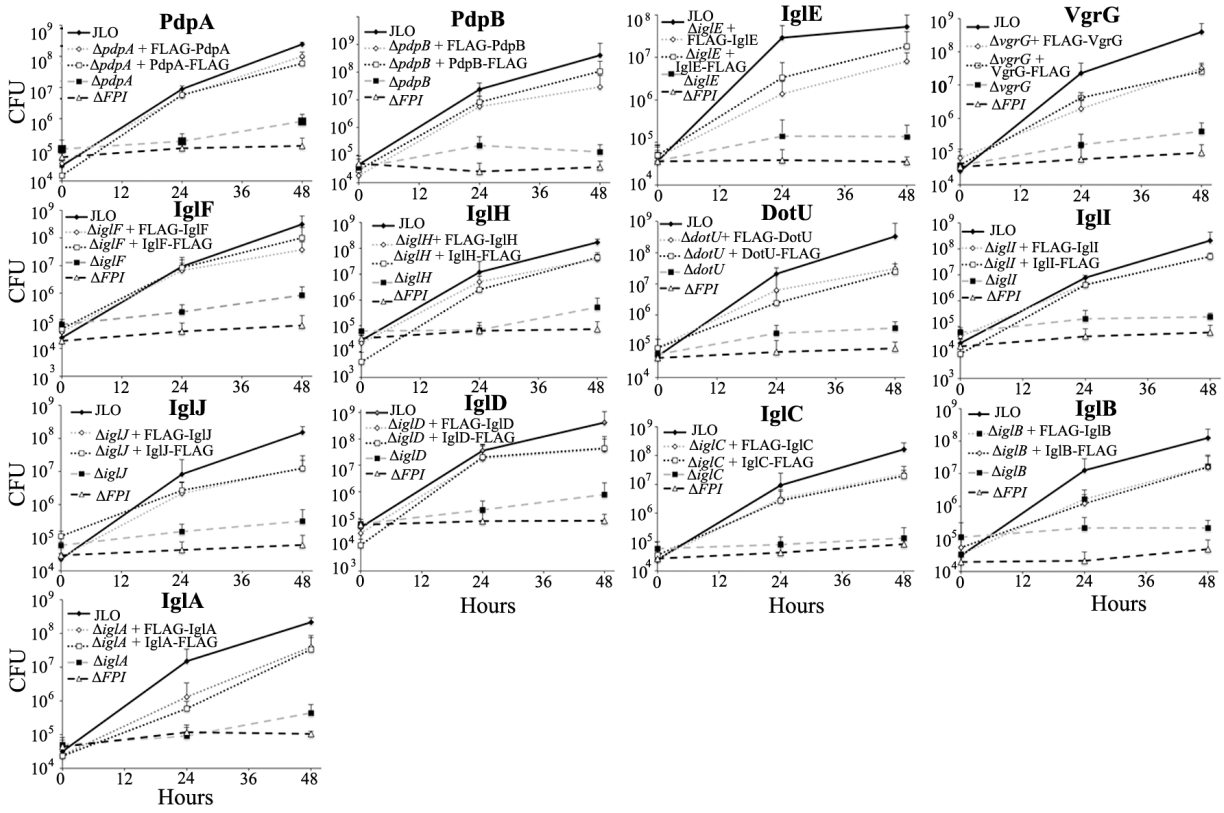

Figure 2.6 Intracellular Growth

Chapter 3: Localization of *Francisella* Pathogenicity Island Encoded Proteins within Infected Macrophage-like Cells[1]

3.1 Abstract

Intracellular pathogens have evolved mechanisms to exploit host cells for their life cycles. Bacterial virulence genes are commonly located within pathogenicity islands. The genome of *Francisella tularensis* encodes a pathogenicity island containing genes for phagosomal escape, intracellular replication, evasion of host immune responses, virulence, and a type VI secretion system. We hypothesize that some *Francisella* pathogenicity island proteins are secreted during infection of host cells. To test this hypothesis, the localization of the *Francisella* pathogenicity island proteins were examined via immuno-fluorescence microscopy within infected macrophage-like cells. Several *Francisella* pathogenicity island encoded proteins (PdpA, IglE, VgrG, IglF, IglG, IglH, DotU, IglI, IglJ, PdpC, PdpE, IglD, IglC, IglB, and IglA) were extracellularly co-localized with the bacteria, but some were localized at some distance from the bacteria (PdpC), and others (PdpB, PdpD, and Anmk) remained inside bacteria. Proteins that were co-localized with bacteria had different patterns of localization. Among the proteins co-localized bacteria was IglC, which surrounded the bacterial cell. IglC is known to be required for phagosomal escape, intracellular growth, and virulence; the localization of IglC was assessed and determined to be dependent on the type VI secretion system. This suggests that some *Francisella* pathogenicity island proteins are secreted while others remain within the bacterium during infection of host cells as structural components of the secretion system and are necessary for secretion.

[1] Hare RF and Hueffer K (2014) Localization of *Francisella* Pathogenicity Island Encoded Proteins within Infected Macrophage-like Cells. Submitted to PLOS ONE.

3.2 Introduction

Pathogenicity islands exist within many pathogenic bacteria, are acquired via horizontal gene transfer, and encode genes that facilitate interactions with host cells [1]. Secretion systems in bacteria involve the transport or translocation of effector molecules from the interior of a bacterial cell through its membranes to the exterior. Protein secretion is an important mechanism for bacteria to adapt and survive in their environment, including within an infected host [2]. Effector proteins are enzymes or toxins that facilitate infection and are secreted by these secretion systems [3].

Francisella tularensis is an intracellular pathogen that possesses the *Francisella* pathogenicity island (FPI) [4]. The FPI is found in all *Francisella* species and strains, and is duplicated in all human-virulent biovars of *F. tularensis*. *F. novicida* and *F. philomiragia* harbor only one copy of the FPI, which makes these species attractive for creating isogenic FPI gene deletion mutants [4, 5]. The molecular mechanisms contributing to the intracellular survival of *Francisella* are still only poorly understood, and FPI mutagenesis approaches are useful in identifying genes required for intracellular replication and virulence [4, 6, 7, 8, 9, 10, 11].

The FPI contains genes with homology to genes encoding type VI secretion systems (T6SS) in other bacteria [12, 13]. Although homology is not conserved for all of the structural components of the characterized T6SSs, the core components possess homologues [14]. Bioinformatics, genetics, biochemical and cell biological approaches have provided evidence that the FPI encodes a functional T6SS [12, 13]. Homologues of *iglA, iglB, iglC, pdpB, dotU,* and VgrG are found in every T6SS identified to date [15, 16]. DotU and PdpB are inner membrane components homologous with the T6SS proteins DotU and IcmF, respectively [15]. IglA, IglB, and IglC are part of the needle that spans bacterial membranes [13, 15]. Two of the

FPI-encoded proteins, IglA and IglB, are IcmF-associated homologous proteins seen in *Rhizobium leguminosarum, Salmonella enterica,* and *Vibrio cholerae* [4, 12, 16, 17, 18]. Mutations in IglA and IglB prevent bacterial escape from the phagosome and inhibit intracellular replication [4, 6, 12, 19, 20]. In some species, these homologues are responsible for secreting proteins, including Hcp and VgrG [16, 18, 21, 22, 23]. Recent studies suggest T6SSs facilitates *Francisella* virulence, intracellular growth, and survival factors; however, the exact molecular mechanisms are uncharacterized [13, 24, 25].

Localizing proteins of the FPI and identifying secreted proteins of *F. tularensis* is important for developing successful intervention strategies. Although the ability of *F. tularensis* to replicate within macrophages is multifactorial, our working hypothesis is that *F. tularensis* secretes FPI-encoded proteins that facilitate the organism's ability to escape the phagosome, enter the cytoplasm to replicate intracellularly, and down regulate the host immune cytokine response. If this is correct, we hypothesize FPI-encoded proteins are secreted during infection within host macrophages. Currently available genetic tools for studying the FPI-encoded proteins consist of green fluorescent proteins (GFP) tags [8] and more recently reporter fusion tag systems [11]. Secretion of FPI-encoded proteins have previously been examined in the *Francisella* live vaccine strain (LVS) with a fusion β-lactamase, however, this system is not applicable to *F. novicida* because *F. novicida* possesses native β-lactamase genes that are secreted and capable of cleaving the fusion tag [11]. In this study, FPI-encoded proteins were expressed as fusion proteins with the small FLAG tag and tracked within infected macrophage-like cells. The localization of IglC in a T6SS mutant was also assessed.

3.3 Materials and Methods

3.3.1 Bacterial and Cell Cultures

Bacterial strains and cell lines used in this study are listed in Table 3.1 *F. novicida* U112 (ATTC® 15482) was cultured aerobically at 37°C on tryptic soy agar (TSA) or in tryptic soy broth (TSB) supplemented with 0.1% cysteine. When selecting for or maintaining transformants, *Francisella* was cultured on TSA containing 15μg/ml of kanamycin. J774-1A murine macrophage-like cells were obtained from the American Type Culture Collection (ATCC®, TIB 67™, BALB/C macrophage). J774 cells were grown in flasks in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO® Invitrogen Grand Island, NY USA) supplemented with 10% newborn calf serum (NCS) and maintained at 37°C in a humidified 6.5% CO_2 incubator. The mosquito hemocyte like cells Sua 1B were grown in Schneider's Insect Medium (Sigma Aldrich® St. Louis, MO USA) supplemented with 20% fetal bovine serum at 28°C and flasks were capped tightly [26].

3.3.2 SDS-PAGE and Western Blotting

SDS-PAGE was performed using standard techniques. Proteins were transferred to Immobilon-P membrane (Millipore Billerica, MA USA), and then blocked in 5% non-fat dry milk (NFDM) in Tris-Buffered Saline and Tween® 20 (Fisher BioReagents® Fair Lawn, NJ US) solution (TBST) containing 1mM Tris, 15mM NaCl, 2mM KCl, and 0.1% Tween® 20 for 1h. To detect FLAG-tagged proteins, the blots were incubated with (1/5,000) monoclonal M2 mouse anti-FLAG® antibodies (Sigma Aldrich® St. Louis, MO USA) in 5% NFDM in TBST. To detect bound antibodies, blots were incubated with (1/5,000) Peroxidase goat Anti-Mouse secondary antibodies (Zymed® Laboratories Invitrogen Immundetection San Francisco, CA USA) in 5%

NFDM in TBST. To visualize protein bands, blots were incubated with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific Rockford, IL USA) prior to exposing and developing film.

3.3.3 Immuno-fluorescence Microscopy

J774 murine macrophage-like cells or Sua-1B mosquito hemocyte-like cells were grown on coverslips and infected with *F. novicida* strains as indicated. Cells were infected for 30min at an MOI of 50, washed with phosphate buffered saline (PBS), and incubated until the desired time point in DMEM containing 10% NCS. Cells were then fixed in 4% paraformaldehyde for 15min at room temperature and rinsed three times with PBS. FLAG-tagged proteins and *Francisella* were detected with monoclonal M2 mouse anti-FLAG® antibodies (Sigma Aldrich®) and rabbit anti-*Francisella novicida*, respectively. All antibodies were diluted (1/500) in PBS containing 0.5% BSA and 0.1% saponin to permeate host cell membranes, while leaving the bacterial cell membranes intact [27]. Primary antibodies were detected with goat anti-mouse and goat anti-rabbit serum conjugated to Alexa Fluor® 488 and 594, respectively (Invitrogen™ MOLECULAR PROBES® Eugene, OR US). DNA was detected with DAPI (Invitrogen™ MOLECULAR PROBES® Eugene, OR US). Coverslips were mounted using Prolong® Gold Antifade reagent (Invitrogen™ MOLECULAR PROBES® Eugene, OR US) and examined using an Olympus TE81 inverted fluorescent microscope with spinning disc confocal capabilities.

Images were collected as Z-stacks and a projection image was generated using the Intelligent Imaging SlideBook™ software package. Exposure time and settings were constant for all slides in each experiment. Using SlideBook™ software, masks were generated for infected macrophage-like cells, bacteria, and FLAG-tagged protein signals. The percentage of bacterial

masks that overlapped with FLAG masks was used to determine the percentage of bacteria associated or co-localized with FLAG-tagged protein. However, this did not account for FLAG-tagged protein that dispersed away from bacteria, therefore the percentage of infected macrophage masks containing FLAG-tagged masks were also determined for every FPI protein with each the C-terminal and N-terminal FLAG tag. At least three independent experiments were performed. The data were analyzed with a left sided Dennett's test comparing each FLAG-tagged proteins' mean to the mean of bacteria not containing a FLAG expressing plasmid for either the percentage of bacteria co-localized with FLAG signal or the percentages of infected macrophage-like cells containing FLAG signal. Significant differences were determined with an $\alpha \leq 0.05$.

3.4 Results

3.4.1 Bacteria Co-Localization with C-terminal FLAG-tagged FPI Proteins During Cell Infection

The localization of FPI-encoded proteins was examined via immuno-fluorescent microscopy of infected murine macrophage-like J774 cells with bacteria expressing C-terminal fusion proteins. The percent of bacteria co-localized with FLAG signal within infected macrophages was determined for all 18 FPI-encoded proteins. At 30min post-infection, bacteria expressing FLAG-tagged IglE, VgrG, IglH, DotU, PdpE, IglD, IglB, and IglA were significantly more often co-localized with FLAG signal compared to control bacteria not expressing epitope-tagged protein ($p \leq 0.038$) (Fig. 3.1A). Bacteria expressing the rest of the FLAG-tagged FPI-encoded proteins were not statistically different from the control bacteria at 30min ($p \geq 0.368$) (Fig. 3.1A). Bacteria expressing FLAG-tagged PdpA, IglE, VgrG, DotU, IglI, IglC, and IglA all

had significantly more bacteria co-localized with FLAG signal compared to control bacteria not expressing epitope-tagged protein ($p \leq 0.008$) at 4h post-infection (Fig. 3.1B). The bacteria expressing the rest of the FPI-tagged proteins were not different from control bacteria, when examining the bacteria co-localized with FLAG signal at 4h ($p \geq 0.814$) (Fig. 3.1B). At 8h into infection, bacteria expressing IglE, IglH, DotU, and IglI had significantly more bacteria co-localized with FLAG signal than the control bacteria ($p \leq 0.029$) (Fig. 3.1C). The other FLAG-tagged FPI-encoded proteins were not different from the controls ($p \geq 0.274$) (Fig. 3.1C). More bacteria were co-localized with FLAG signal from tagged IglE, DotU, PdpE, and IglA ($p \leq 0.012$) with bacteria expressing those tagged proteins compared to control bacteria at 12h, while bacteria expressing the other FPI-tagged proteins were not different than the control bacteria ($p \geq 0.129$) (Fig. 3.1D).

3.4.2 C-terminal FLAG-tagged FPI Protein Localization within Infected Cells

The localization of C-terminal FLAG-tagged FPI proteins were alternatively assessed by calculating the percent of infected cells containing FLAG signal to account for proteins that were secreted into the infected cells but did not co-localize with the bacteria expressing the epitope tagged proteins. Within 30min of infection, the cells infected with bacteria expressing tagged PdpA, IglE, VgrG, IglH, DotU, IglI, PdpC, PdpE, IglD, IglC, IglB, IglA PdpD, and Amnk all had significantly more infected cells containing fluorescent signal when compared to the control cells infected with bacteria not expressing FLAG-tagged proteins ($p \leq 0.036$) (Fig. 3.2A). Also at 30min into infection, the cells infected with bacteria expressing tagged PdpB and IglF were not different from cells infected with control bacteria ($p \geq 0.232$) (Fig. 3.2A). At 4h post infection,

cells infected with bacteria expressing tagged IglE, VgrG, IglH, DotU, IglI, and IglI all had significantly more infected cells containing fluorescent signal ($p \leq 0.029$) than the cells infected with bacteria not expressing FLAG-tagged proteins. However, the cells infected with tagged PdpB, IglF, IglG, IglJ, IglC, IglB, and Anmk were not significantly different from cells infected with bacteria not expressing FLAG-tagged proteins ($p \geq 0.208$) (Fig. 3.2B). When comparing the amount of infected cells containing FLAG signal at 8h after infection, the infected cells that expressed PdpA, IglE, VgrG, IglG, DotU, IglI, IglF, IglJ, PdpC, PdpE, IglD, IglC, IglB, and IglA had significantly more infected cells containing FLAG signal ($p \leq 0.009$). The cells infected with bacteria expressing tagged PdpB and IglG were not different from the control cells containing bacteria not expressing FLAG-tagged proteins ($p \geq 0.142$) (Fig. 3.2C). More infected cells contained FLAG signal at 12h within cells infected with bacteria expressing tagged PdpA, IglE, VgrG, DotU, IglJ, PdpC, PdpE, IglD, IglC, IglB, IglA, and Anmk ($p \leq 0.042$). Additionally at 12h, cells infected with bacteria expressing FLAG-tagged PdpB, IglF, IglG, IglH, and PdpD were not statistically different ($p \geq 0.103$) when compared to cells infected with bacteria not expressing FLAG-tagged proteins (Fig. 3.3D).

3.4.3 Bacteria Co-Localization with N-terminal FLAG-tagged FPI Proteins During Cell Infection

The localization of FPI-encoded proteins was also examined via immuno-fluorescent microscopy of infected murine macrophage-like cells with bacteria expressing N-terminal fusion proteins in order to assess effects of tags on the termini of FPI-encoded proteins. The percent of bacteria co-localized with FLAG signal within infected macrophages was determined for all 18 FPI proteins with a N-terminus FLAG-tag. At 30min post-infection, bacteria expressing FLAG-

tagged IglE, VgrG, DotU, IglI, and PdpC had significantly more bacteria co-localized with FLAG signal compared to bacteria not expressing epitope-tagged protein ($p \leq 0.47$) (Fig. 3.3A). The bacteria expressing the other tagged proteins at 30min were not statistically different from the control bacteria not expressing tagged protein ($p \geq 0.166$) (Fig. 3.3A). Bacteria expressing tagged IglI, PdpC, and IglD had significantly more bacteria co-localized with FLAG signal compared to cells infected with control bacteria not expressing epitope-tagged protein ($p \leq 0.021$) at 4h post-infection (Fig. 3.3B). Additionally at 4h into infection, bacteria expressing the rest of the FPI-tagged proteins were not different from control bacteria when examining the bacteria co-localized with FLAG signal ($p \geq 0.347$) (Fig. 3.3B). Bacteria expressing tagged IglE, DotU, and IglI, had significantly more bacteria co-localized with FLAG signal than cells infected with the control bacteria at 8h post infection ($p \leq 0.037$) (Fig. 3.3C). Bacteria expressing the other FPI proteins were not different from the control ($p \geq 0.161$) (Fig. 3.3C). More bacteria were co-localized with FLAG signal from tagged IglE, VgrG, DotU, IglI, PdpC, IglC, and IglA ($p \leq 0.032$) when compared to control bacteria at 12h, while bacteria expressing the other FPI-tagged proteins were not different than the control bacteria ($p \geq 0.115$) (Fig. 3.3D).

3.4.4 N-terminal FLAG-tagged FPI Protein Localization within Infected Cells

Similar to the analysis of C-terminally tagged proteins, the localization of FPI proteins with N-terminal FLAG tags were also assessed by calculating the percent of infected cells containing FLAG signal. At 30min into the infection, cells infected with bacteria expressing tagged PdpA, IglE, DotU, IglI, PdpC, and IglA all had significantly more infected cells containing FLAG signal when compared to control cells infected with bacteria not expressing

FLAG-tagged proteins ($p \leq 0.008$) (Fig. 3.4A). Cells infected with bacteria expressing tagged PdpB VgrG, IglG, IglJ, PdpE, IglC, PdpD, and Amnk were not significant at 30min ($p \geq 0.135$) (Fig. 3.4A). Cells infected with bacteria that expressed tagged IglE, PdpC, IglD, and IglC all had significantly more infected cells containing FLAG signal ($p \leq 0.045$) than cells infected with bacteria not expressing FLAG-tagged proteins at 4h post-infection (Fig. 3.4B). However the cells infected with bacteria expressing tagged PdpB, VgrG, IglG, IglH, DotU, IglF, IglJ, IglB, IglA, PdpD, and Anmk were not significantly different from the cells infected with bacteria not expressing FLAG-tagged proteins at 4h ($p \geq 0.173$) (Fig. 3.4B). Also at 8h, cells infected with bacteria expressing PdpA, PdpB, VgrG, IglF, IglG, IglI, IglJ, PdpE, IglC, IglB, IglA, PdpD, and Anmk were not different ($p \geq 0.132$) than infected cells containing bacteria not expressing FLAG-tagged proteins (Fig. 3.4C). Not any of the cells infected with bacteria expressing the different FLAG-tagged proteins were significant for containing FLAG at 12h into the infection (Fig. 3.4D).

3.4.5 Localization of FPI-Encoded Proteins within Infected Cells

FPI proteins with consistent FLAG detection had varying patterns of distribution of FLAG signal when compared to each other within infected murine macrophage-like cells (Fig. 3.5) and mosquito hemocyte-like cells (Fig. 3.6). IglA was localized at the poles of bacteria, either unipolar or bi-polar (Fig. 3.5, Fig. 3.6, and S. Fig. 3.1). IglC, VgrG, and IglE were co-localized with bacteria that surrounded and expanded beyond bacteria (Fig. 3.5 and Fig. 3.6). IglD and PdpA were also localized at the poles of bacteria, either unipolar or surrounding the bacteria with the exception of one pole of bacteria (Fig. 3.5 and Fig. 3.6). The C-FLAG PdpE was studded around the bacterium, while the N-terminal tagged protein was not detected by

immuno-fluorescent microscopy (Fig. 3.6 and S. Fig. 3.3). PdpC was also detected both co-localized with bacteria and dispersing away from the bacteria (Fig. 3.6). IglI was distinctly localized to the bacterium; it surrounded the bacterium uniformly (Fig. 3.5, Fig. 3.6, and S. Fig. 3.2).

3.4.6 Localization is Dependent on PdpB

Less FLAG was detected when IglC was expressed by the Δ*pdpB* mutant when compared to expression of tagged IglC by wild type bacteria (Fig. 3.7). Over 20% of wild type bacteria were associated with IglC, while less than 7% of Δ*pdpB* bacteria were associated with IglC (Fig. 3.7B). The differences between IglC association with wild type or Δ*pdpB* mutant bacteria were statistically significant. IglC was examined by Western blot to determine if the mutant expressed the tagged proteins. IglC-FLAG was expressed at similar levels in the wild type and Δ*pdpB* mutant backgrounds (Fig. 3.7C). Therefore, extracellular co-localization of IglC with bacteria is dependent on the T6SS.

3.5 Discussion

Genes within the FPI are required for type VI secretion, intracellular growth, and virulence [13, 24, 25, 28]. Many of the FPI-encoded proteins are part of a T6SS, therefore we hypothesized that some of the FPI-encoded proteins would be directed for secretion by that secretion system, such as effector proteins or chaperones [11, 28]. A recent study that utilized a fusion Temoniera (TEM) β-lactamase reporter in LVS identified IglE, IglC, VgrG, IglI, PdpE, PdpA, IglJ, and IglF as secreted proteins, and determined that secretion is dependent on the core

components of the T6SS: DotU, VgrG, IglC, and IglG [12]. Previously another study used a CyaA reporter to show IglI and VgrG are secreted in both LVS and *F. novicida* [28].

Although these studies were the first to identify secreted FPI-encoded proteins, there were some limitations to the tools used in those experiments. The TEM β-lactamase reporters have adverse effects including low levels of protein expression; therefore results were only based on one time point at 18h post-infection [11]. In addition to low expression, the TEM β-lactamase expresses functionless proteins that are unable to complement an intracellular growth phenotype [11]. Since *F. novicida* possesses native β-lactamase genes that are secreted and capable of cleaving the fusion tag, the TEM β-lactamase assay is not applicable for this subspecies [11]. In the present study, we examined the localization of FPI proteins in *F. novicida* during infection of macrophage-like cells. We used *Francisella* expression plasmids that express all 18 of the FPI-encoded proteins from *F. novicida* with a C-terminal epitope FLAG tag as well as a N-terminal epitope FLAG tag. These tagged FPI proteins encode functional proteins that complement intracellular growth of isogenic mutants (see Chapter 2).

To help discern the function of FPI-encoded proteins, their localization within host cells was examined at different time points during infection. These time points were chosen according to *Francisella's* intracellular life cycle [20, 29, 30, 31]. By 30min post-infection, internalized bacteria escape the phagosome and enter the host cell's cytoplasm. Bacteria replicate intracellularly by 4h post-infection. By 12h post-infection bacteria manipulate host cells by avoiding immune responses and initiating autophagy.

Macrophage-like cells were infected with wild type bacteria containing the *Francisella* expression plasmids. Immuno-fluorescence microscopy revealed different FPI proteins that exhibit different patterns of FLAG patterns of localization. Using the *Francisella* expression

plasmids in *F. novicida,* PdpA, IglE, VgrG, IglF, IglG, IglH, DotU, IglI, IglJ, PdpC, PdpE, IglD, IglC, IglB, and IglA were localized within macrophage-like cells on the outside of bacterial cell membranes. Proteins that were consistently detected in the cytoplasm of host cells were VgrG, IglI, IglE, IglD, IglC, IglB, and IglA (Fig. 3.1-3.4). The detection of other proteins (PdpA, PdpC, PdpE, IglF, IglG, IglH, IglJ and DotU) varied depending on time point. There was a high degree of variation in the localization of N-terminal tagged proteins at 12h. Specifically, FLAG-tagged IglE, VgrG, DotU, PdpC, IglC, and IglA were significant when assessed for bacteria FLAG co-localization while none were significant when the assessed for infected macrophages containing FLAG. This can be explained by the fact that more area was assessed in the cell level analysis increasing total non-specific background fluorescence compared to bacterial association. Also, since the N terminal is translated first, the FLAG tag may alter protein folding and localization. It is not surprising that N-terminally tagged PdpE was not expressed, as PdpE is predicted to have an export signal sequence on the N-terminus [11].

Some proteins expressed were not detected in the cytoplasm of host cells. Localization of PdpB, PdpD, and Anmk possessing FLAG tags were consistently similar to that of wild type cells in bacteria, and within infected host cells, however, these proteins were verified by Western blott for expression. This suggests PdpB, PdpD, and Anmk were not secreted from bacteria, which is consistent with other studies [13, 32].

The current model for the T6SS in *Francisella* suggests PdpB is a transmembrane anchor protein, which spans the inner-membrane and extends into the periplasmic space [13]. Our inability to detect PdpB confirms the appropriateness of using saponin-permeabilized cells to detect FLAG epitopes outside of bacteria while leaving the bacterial cell wall [27]. It is interesting that this microscopy study detected DotU as having a strong extracellular signal.

DotU is an inner-membrane component of the secretion system of *Francisella* and all T6SS's [13, 33]. Solubility properties have identified DotU as predominantly membrane-associated, partially soluble, and localized to the inner-membrane and periplasmic space where it stabilizes the secretion system [13, 33]. The localization of DotU has not been visualized before; it is interesting that in this study, microscopy detected DotU extracellularly. DotU could be temporarily exposed to the extracellular space of bacterial cells during the contraction of the tube of the secretion system as proteins are secreted.

The inner tube of the T6SS is speculated to be a polymer of IglC, which lies within the IglA and IglB polymer that contracts and drives IglC through the host cell membrane [13]. This contraction of IglA and IglB, could temporarily expose components of the secretion system (IglA, IglB, IglC, DotU, and potentially other proteins) to extracellular staining. Also, IglA-IglB polymers span both the inner- and outer-membrane of *Francisella*, and thus are exposed extracellularly but not necessarily secreted. VgrG and PdpE are located on the point of the secretion channel-forming tube and would, therefore, appear outside of bacteria, as shown in this study (Fig. 3.5 and Fig. 3.6) [13].

Several secreted proteins were identified in this study that have previously been identified as secreted from LVS, including PdpA, IglE, VgrG, IglF, IglI, IglJ, IglC, and PdpE [11, 28, 34]. This study also identified DotU, IglA, IglB, IglD, IglG, IglH, and PdpC as being localized to the outside of bacteria within infected host cells in addition to PdpA, IglE, VgrG, IglF, IglI, IglJ, IglC, and PdpE.

DotU, PdpB, IglA, IglB, IglC, VgrG, PdpE, IglE, are components of the T6SS, and they have extracellular localization with the exceptions of PdpB and DotU. IglF, IglG, IglH, IglJ, PdpD, and AnmK were not significant at most time points examined compared to IglI, IglD,

PdpA, and PdpC. It is not clear whether these proteins are secreted, localized to the outer membrane of *Francisella*, or temporarily localized to the outer membrane as components of the secretion system during transport of other secreted proteins. An alternative explanation is their detection in this study is due to over expression from the *Francisella* expression plasmids.

To further examine localization of IglC, the IglC-FLAG plasmid was transformed into Δ*pdpB* to test if localization was dependent on the T6SS. PdpB is homologous to IcmF, which is an inner membrane component of the T6SS in *V. cholera* [14]. PdpB was not detected through microscopy because it is an inner membrane protein of *Francisella* (Fig. 3.1-3.4) [13]. The co-localization of IglC-FLAG with bacterial cells was significantly lower in Δ*pdpB* bacteria compared to wild type cells (Fig. 3.7B). The expression of IglC was examined by Western blot to determine if the mutant was expressing IglC-FLAG, and both wild type and Δ*pdpB* expressed IglC at similar levels (Fig. 3.7C). This further supports the hypothesis that the FPI encodes a T6SS and that proteins are secreted through the secretion system.

These results further suggest some FPI proteins are secreted in *Francisella novicida,* while others remain associated with the bacterium during infection of host cells and some of those are necessary for secretion. Although there are differences among the *Francisella species*, the FPI-encoded proteins are conserved among the *Francisella species* both in their sequences and functions in intracellular growth, virulence, and the T6SS. Due to the similarities of the T6SS and its secreted proteins between LVS and *F. novicida,* this study confirms *F. novicida* as a valuable model to study the molecular mechanism employed by *F. tularensis* during infection of host cells.

3.6 Authors' Contributions

RFH constructed epitope-tagged plasmids, performed Western blots, growth assays, and microscopy experiments. KH and RFH designed the study. RFH and KH wrote the manuscript.

3.7 Acknowledgements

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3.8 References

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3.9 Chapter 3 Figure Legends

3.9.1 Figure 3.1 Bacteria Associated with FPI C-tagged Proteins

The percent of bacteria associated with C-terminal FLAG-tagged FPI proteins within J774 cells at various time-points during infection is shown. This graph represents the mean of three independent experiments. Error bars indicate the standard deviations. Asterisks indicate significance ($p \leq 0.05$) when compared to the no plasmid wild type control.

3.9.2 Figure 3.2 Infected Macrophages with FPI C-tagged Proteins

The percent of infected macrophages containing C-terminal FLAG-tagged FPI proteins within J774 cells at various time-points during infection is shown. This graph represents the

mean of three independent experiments. Error bars indicate the standard deviations. Asterisks indicate significance ($p \leq 0.05$) when compared to the no plasmid wild type control.

3.9.3 Figure 3.3 Bacteria Associated with FPI N-tagged Proteins

The percent of bacteria associated with N-terminal FLAG-tagged FPI proteins within J774 cells at various time-points during infection is shown. This graph represents the mean of three independent experiments. Error bars indicate the standard deviations. Asterisks indicate significance ($p \leq 0.05$) when compared to the no plasmid wild type control.

3.9.4 Figure 3.4 Infected Macrophages with FPI N-tagged Proteins

The percent of infected macrophages containing N-terminal FLAG-tagged FPI proteins within J774 cells at various time-points during infection is shown. This graph represents the mean of three independent experiments. Error bars indicate the standard deviations. Asterisks indicate significance ($p \leq 0.05$) when compared to the no plasmid wild type control.

3.9.5 Figure 3.5 Localization of FPI Proteins within Infected Macrophages

J774 cells were infected with wild type bacteria without a plasmid and wild type containing the *Francisella* expression plasmids that express PdpA, IglE, VgrG, IglI, IglD, IglC, IglB and IglA with either C-terminal FLAG tag (left) or N-terminal FLAG tag (right) at 4h post-infection. Middle columns and the red in the merged right columns indicate bacteria. Left columns and the green in the merged right columns indicate FLAG-tagged proteins. Host cell nuclei are indicated by blue in merged right columns.

3.9.6 Figure 3.6 Localization of FPI Proteins During Infection of Sua-1B Cells

J774 cells infected with wild type containing the *Francisella* expression plasmids that express IglA, IglC, IglI, and PdpE for 4h (A). Sua-1B cells infected with wild type containing the *Francisella* expression plasmids that express IglA, IglC, IglI, and PdpE for 4h (B). Middle columns and red in the right column indicate bacteria. C-terminally FLAG-tagged proteins are shown in the left columns and are also green in the right column. Host cell nuclei are displayed as blue in the merged, right columns.

3.9.7 Figure 3.7 IglC Secretion is Dependent on T6SS

Microscopy of IglC-FLAG expressed in both wild type and Δ*pdpB* strains of U112 within J774 cells at 4h post infection (A). Automated image analysis of bacterial cells associated with FLAG signal in wild type and *ΔpdpB* backgrounds shown in (B). Each bar represents the mean from three independent experiments of the percent of bacteria associated with FLAG signal, and error bars represent the standard deviation. An asterisk indicates the value was significantly different from that of the wild type in a Two-tailed t test ($p \leq 0.05$). Western blot shows FLAG expression of IglC-FLAG in wild type and Δ*pdpB* strains of U112 (C).

3.9.8 S. Figure 3.1 Three-dimensional Reconstruction of IglA

Three-dimensional reconstructions were comprised from a series of images that were taken through the macrophage cell infected with wild type containing *Francisella* expression plasmids. Bacteria are red, FLAG-tagged protein is blue, and host cell nuclei are blue.

3.9.9 S. Figure 3.2 Three-dimensional Reconstruction of IglI

Three-dimensional reconstructions were comprised from a series of images that were taken through the macrophage cell infected with wild type containing *Francisella* expression plasmids. Bacteria are red, FLAG-tagged protein is blue, and host cell nuclei are blue. IglI completely surrounds bacteria.

3.9.10 S. Figure 3.3 Three-dimensional Reconstruction of PdpE

Three-dimensional reconstructions were comprised from a series of images that were taken through the macrophage cell infected with wild type containing *Francisella* expression plasmids. Bacteria are red, FLAG-tagged protein is blue, and host cell nuclei are blue.

Chapter 3 Tables

Table 3.1 List of Strains and Plasmids

Strain	Description
J774-1A	Murine Macrophages cell lines
Sua-1B	Mosquito hemocytes cell lines [26]
F. novicida U112	*Francisella novicida* prototype strain (ATTC)
F. novicida U112 + pKH22	PdpA-FLAG
F. novicida U112 + pKH40	FLAG-PdpA
F. novicida U112 + pKH24	PdpB-FLAG
F. novicida U112 + pKH50	FLAG-PdpB
F. novicida U112 + pKH9	IglE-FLAG
F. novicida U112 + pKH34	FLAG-IglE
F. novicida U112 + pKH10	VgrG-FLAG
F. novicida U112 + pKH35	FLAG-VgrG
F. novicida U112 + pKH26	IglF-FLAG
F. novicida U112 + pKH44	FLAG-IglF
F. novicida U112 + pKH11	IglG-FLAG
F. novicida U112 + pKH36	FLAG-IglG
F. novicida U112 + pKH12	IglH-FLAG
F. novicida U112 + pKH45	FLAG-IglH
F. novicida U112 + pKH13	DotU-FLAG
F. novicida U112 + pKH37	FLAG-DotU
F. novicida U112 + pKH14	IglI-FLAG
F. novicida U112 + pKH39	FLAG-IglI
F. novicida U112 + pKH15	IglJ-FLAG
F. novicida U112 + pKH47	FLAG-IglJ
F. novicida U112 + pKH5	PdpC-FLAG
F. novicida U112 + pKH41	FLAG-PdpC
F. novicida U112 + pKH16	PdpE-FLAG
F. novicida U112 + pKH38	FLAG-PdpE
F. novicida U112 + pKH6	IglD-FLAG
F. novicida U112 + pKH48	FLAG-IglD
F. novicida U112 + pKH4	IglC-FLAG
F. novicida U112 + pKH46	FLAG-IglC
F. novicida U112 + pKH18	IglB-FLAG
F. novicida U112 + pKH43	FLAG-IglB
F. novicida U112 + pKH8	IglA-FLAG
F. novicida U112 + pKH27	FLAG-IglA
F. novicida U112 + pKH7	PdpD-FLAG
F. novicida U112 + pKH42	FLAG-PdpD
F. novicida U112 + pKH25	AnmK-FLAG
F. novicida U112 + pKH49	FLAG-AnmK
Δ*pdpB* + pKH4	*F. novicida* JLO *ΔpdpB* + IglC-FLAG

Chapter 3 Figures

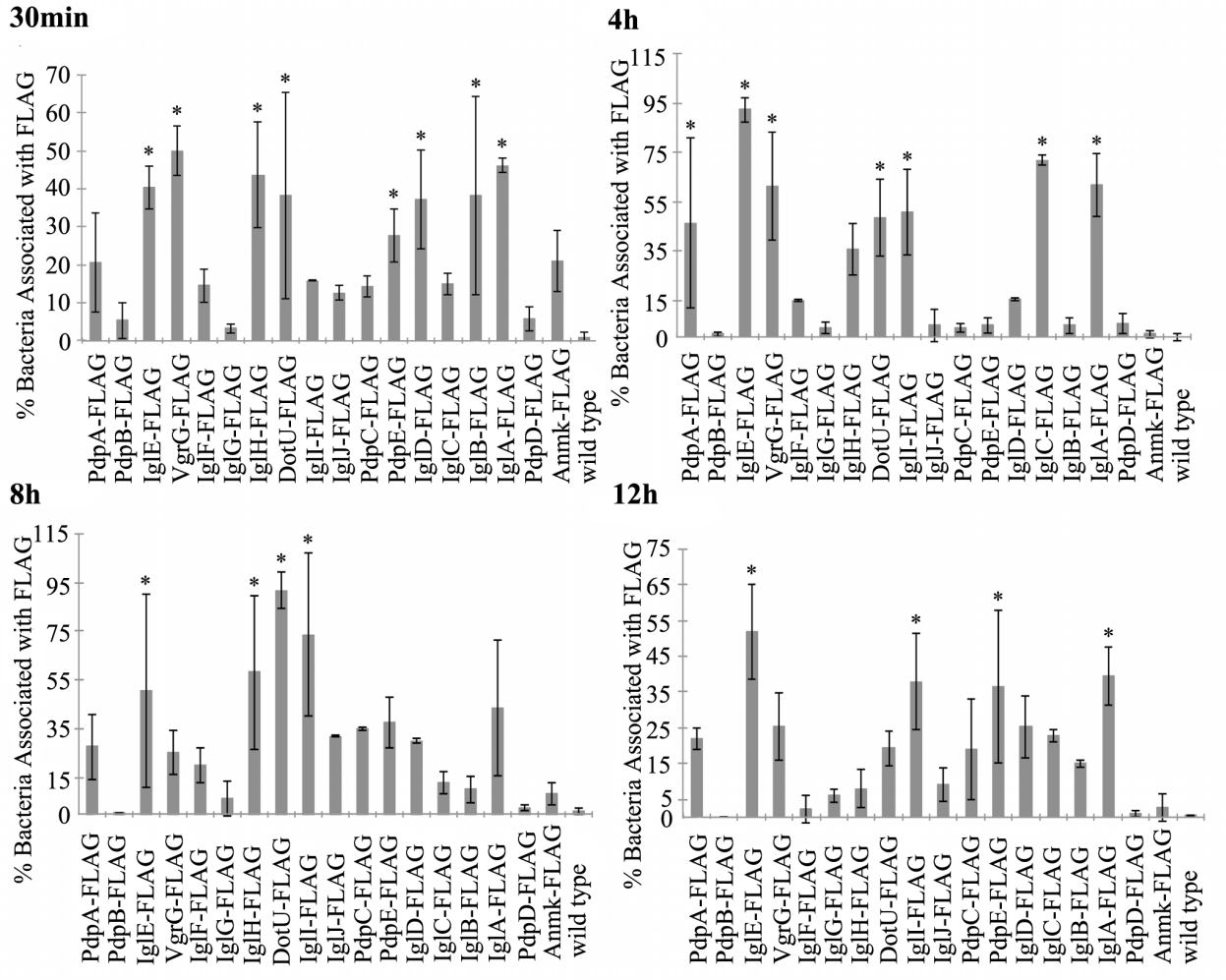

Figure 3.1 Bacteria Associated with FPI C-tagged Proteins

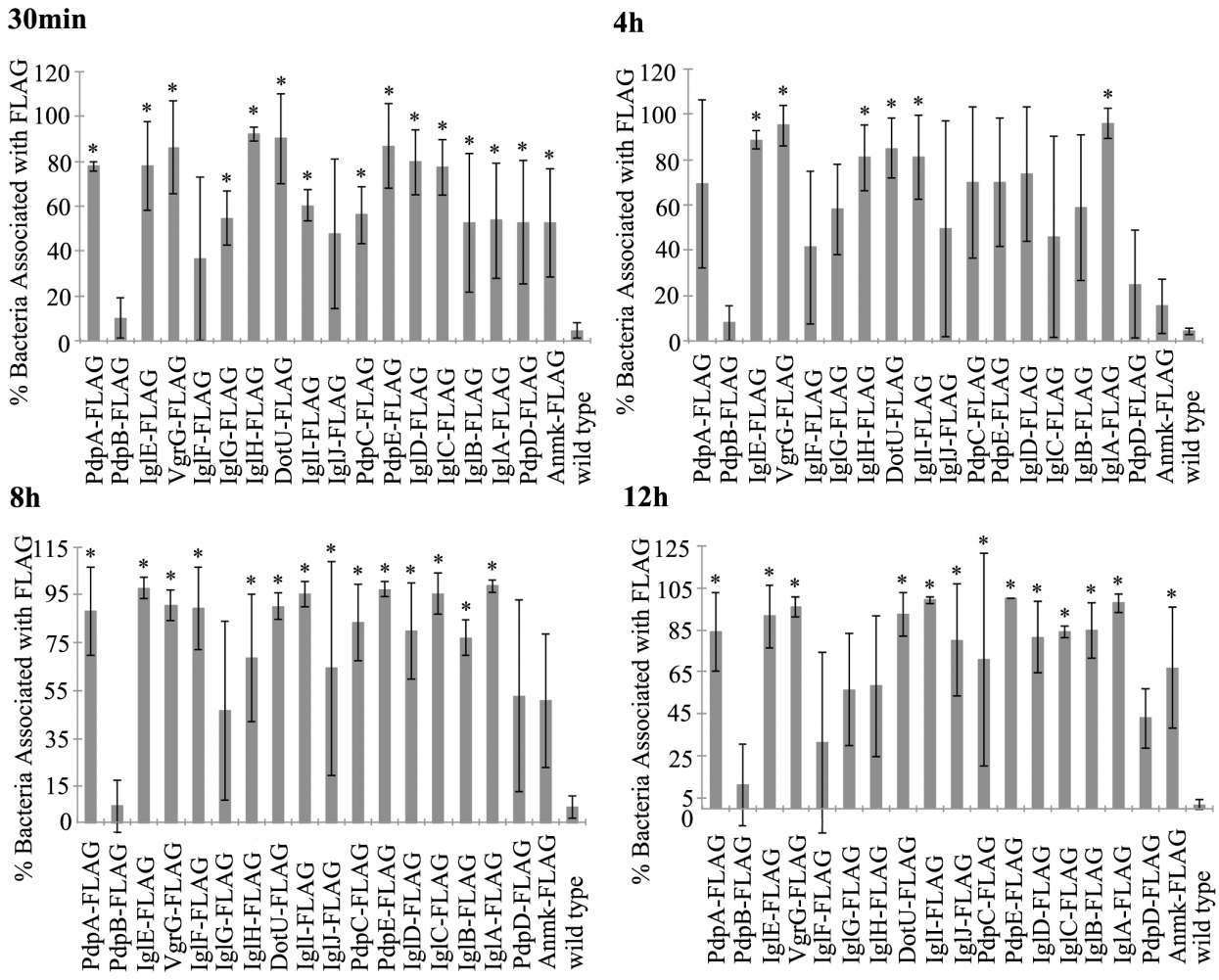

Figure 3.2 Infected Macrophages with FPI C-tagged Proteins

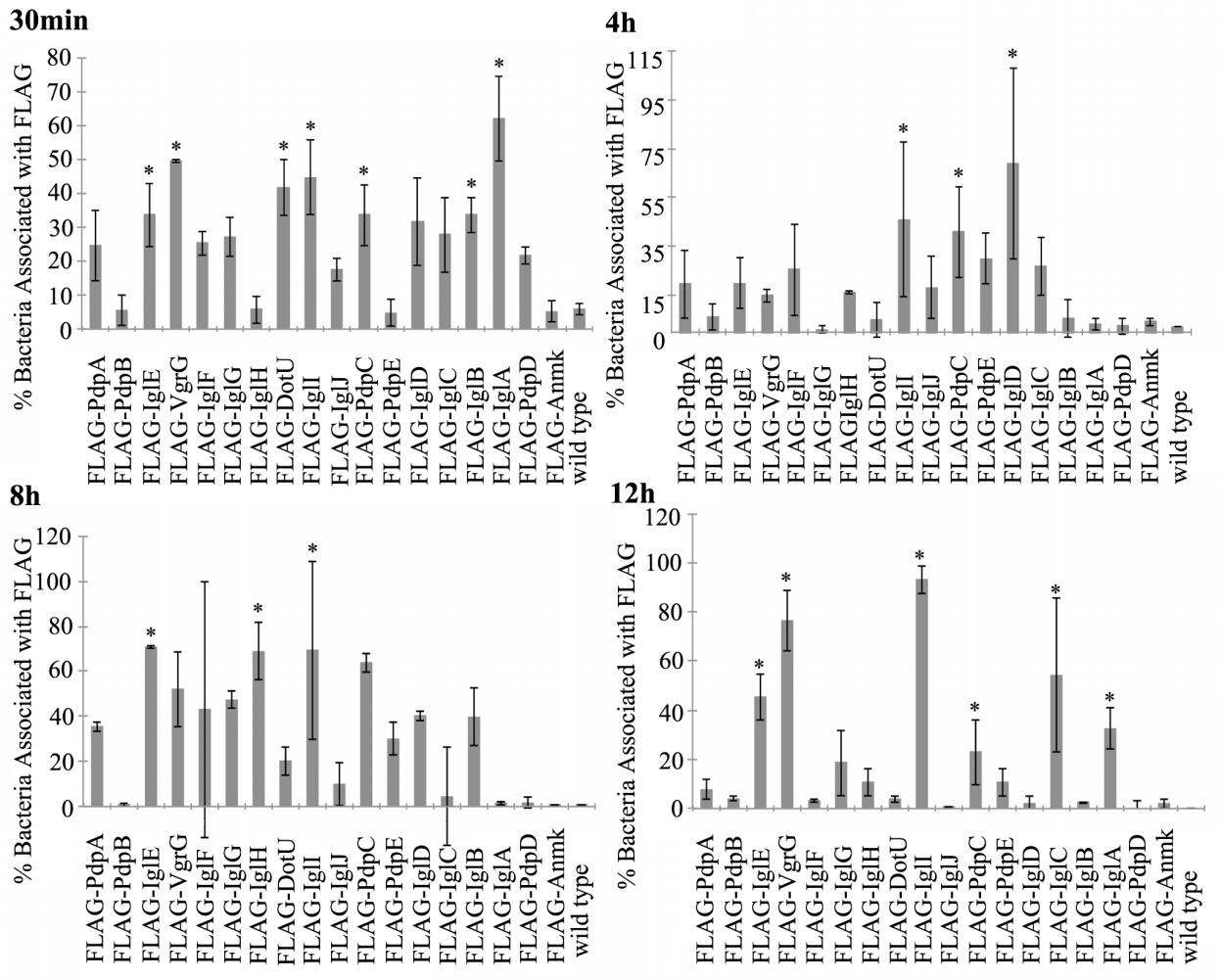

Figure 3.3 Bacteria Associated with FPI N-tagged Proteins

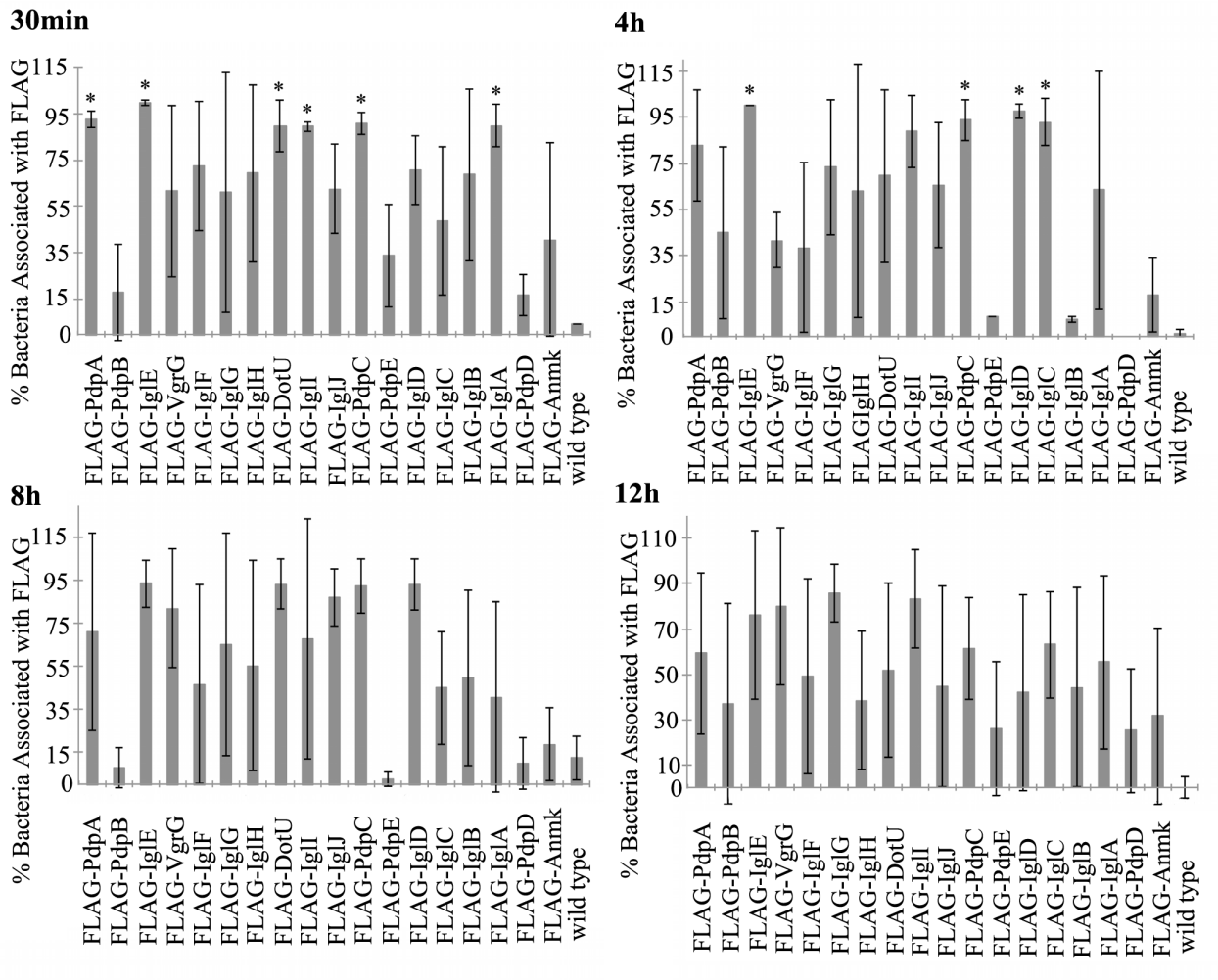

Figure 3.4 Infected Macrophages with FPI N-tagged Proteins

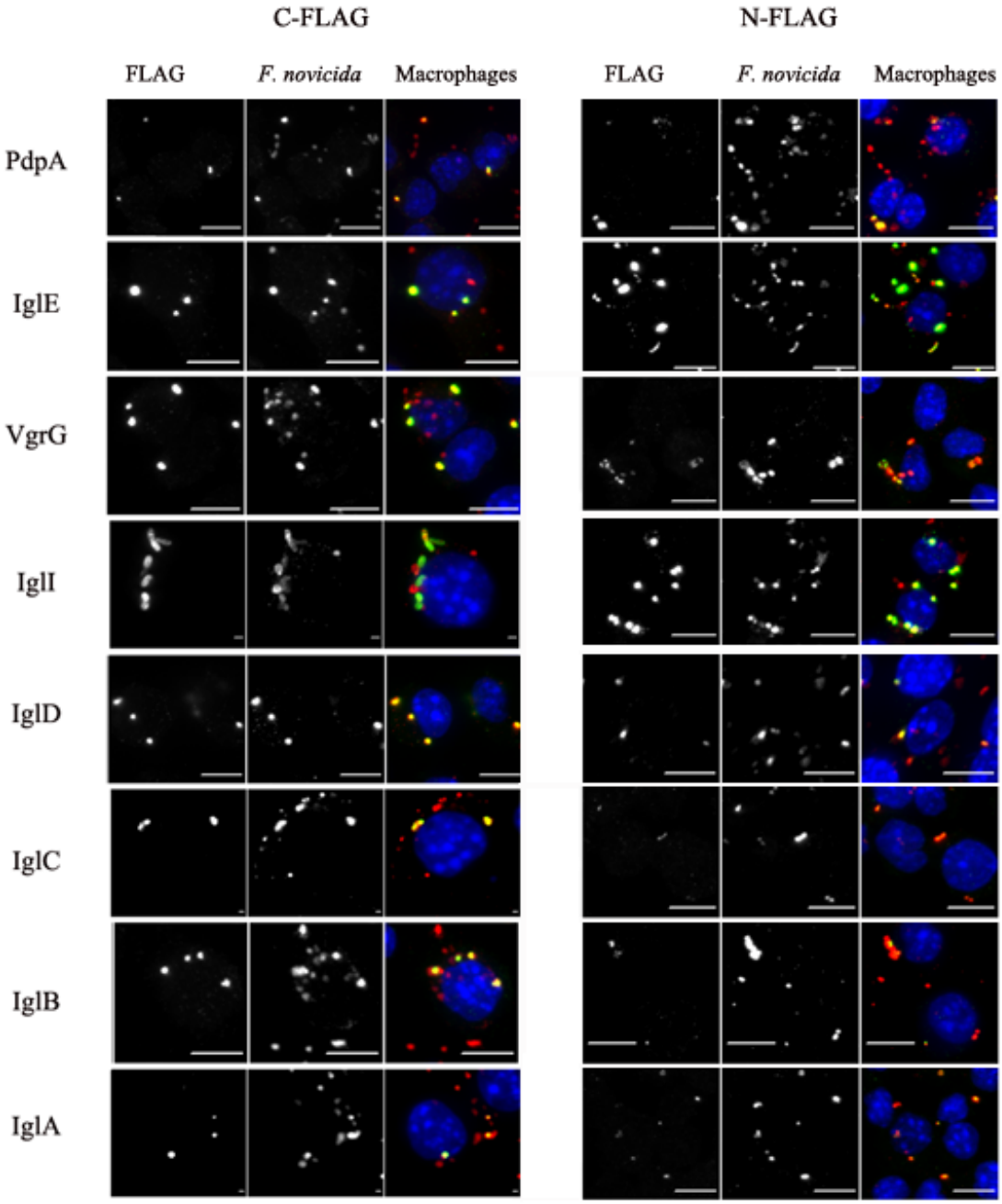

Figure 3.5 Localization of FPI Proteins within Infected Macrophages

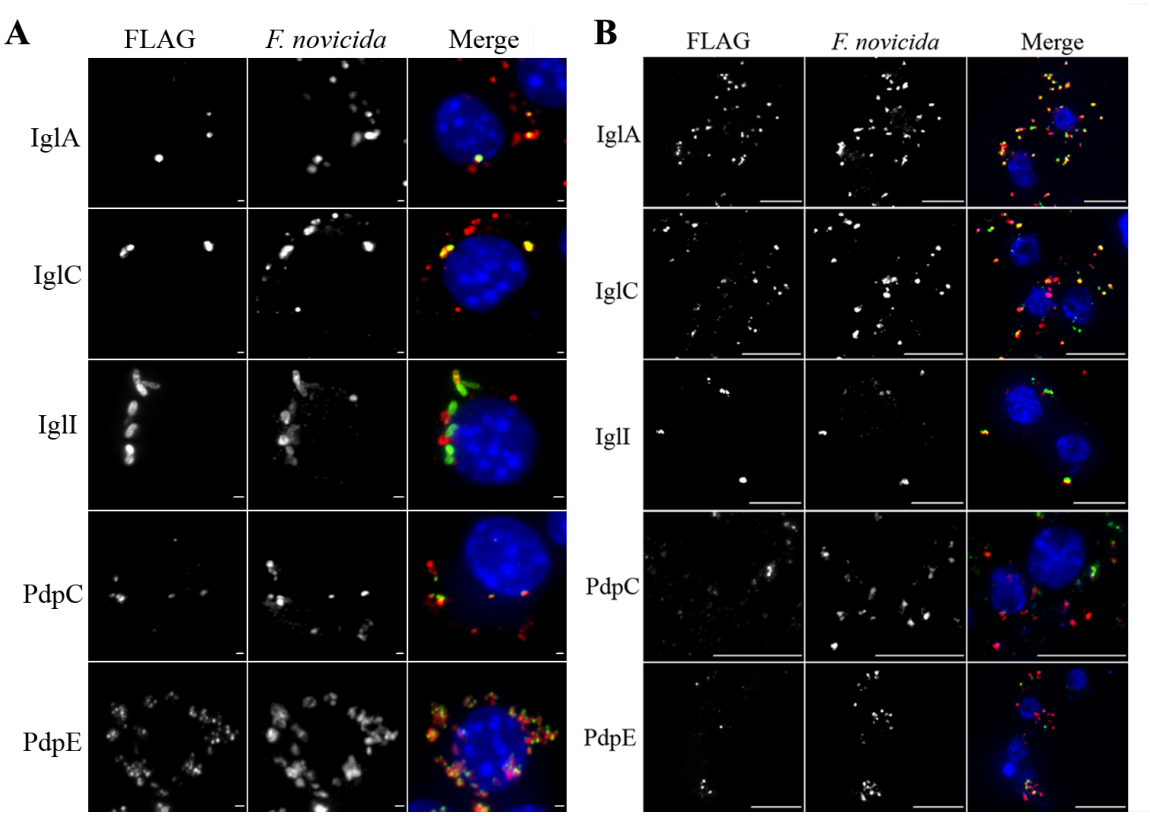

Figure 3.6 Localization of FPI Proteins During Infection of Sua-1B Cells

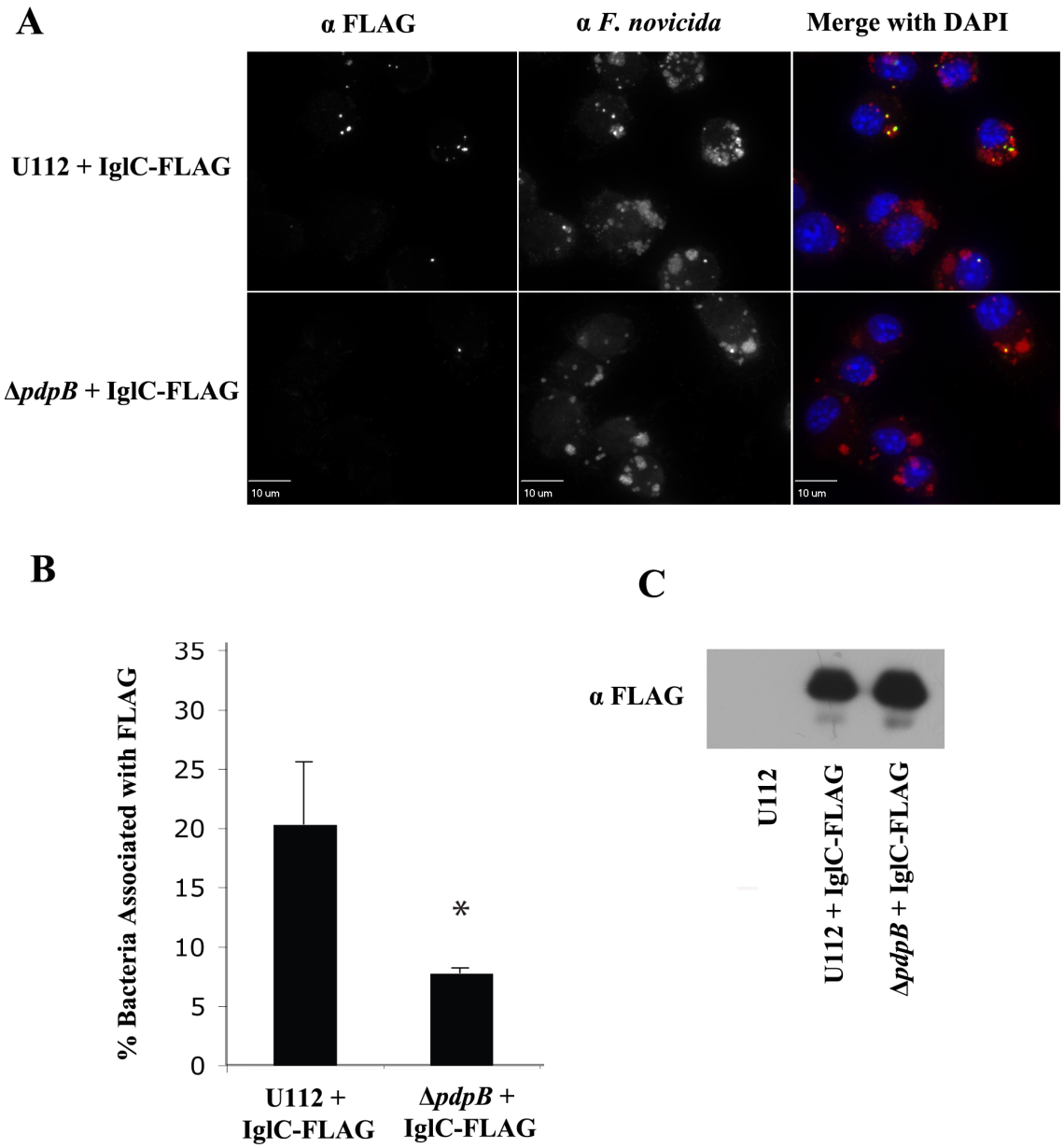

Figure 3.7 IglC Secretion is Dependent on T6SS

Chapter 4: PdpC is a Secreted Protein of *Francisella novicida*[1]

4.1 Abstract

Intracellular bacteria alter the function of host cells through secretion of effector proteins that interfere with functions of the infected cells. *Francisella tularensis*, a Category A select agent of bioterrorism, encodes a type VI secretion system in the *Francisella* pathogenicity island. Several of the proteins encoded within this genetic element are required for virulence in animal models and growth in host cells. *pdpC* is the largest open reading frame in the *Francisella* pathogenicity island, has not been characterized in intracellular growth or virulence, is not a component of the secretion system, and is localized within infected macrophages. Therefore, we hypothesize that PdpC is an effector protein secreted through the type VI secretion system. In this study we found that *Francisella novicida* PdpC was required for full virulence in mice and embryonated chicken eggs. PdpE, the gene downstream of PdpC, was not required for virulence in mice or embryonated chicken eggs. Microscopy of infected cells showed PdpC was localized in close proximity with, and dispersing away from bacteria, while PdpE was localized to the bacteria in a studded configuration. The localization of both PdpC and PdpE within host cells was dependent on PdpB, a homologue of a canonical component of the type VI secretion apparatus.

[1] Hare RF, Nix EB, Cheung KM, Schmerk CL, Chou AY, Elkins KL, Nano FE and Hueffer K (2014) PdpC is a potentially secreted protein of *Francisella novicida* is prepared for submission to PLOS ONE.

4.2 Introduction

Intracellular bacteria, especially those surviving and replicating within macrophages, often evade cellular defence mechanisms. One mechanism to interfere with the function of infected cells is to secrete proteins into the host cell cytoplasm. Some intracellular pathogens have evolved intricate secretion systems that can translocate so-called effector proteins into the host cell. Well-studied examples of such secretion systems include the type III secretion system of *Salmonella* and *Shigella*, as well as the type IV secretion system in *Legionella* [1,2]. Currently eight types of secretion systems have been described [3, 4]. The presence of such secretion systems often distinguishes virulent isolates from closely related avirulent species.

A type VI secretion system (T6SS) has recently been identified in *F. tularensis* [5, 6, 7]. *Francisella tularensis* is a facultative intracellular, gram-negative bacterial pathogen that causes the acute febrile disease tularemia [8]. There are two pathogenic *F. tularensis* biotypes. *F. tularensis* subsp. *tularensis* ("type A") is found only in North America while *F. tularensis* subsp. *holarctica* ("type B") is found throughout the Northern hemisphere. There are conflicting data concerning the relative human virulence of the different *F. tularensis* subspecies [9]. They are all highly infectious relative to other bacterial pathogens. *F. novicida* is similar at the genomic level to *F. tularensis* [10]; it is not infectious for immunocompetent humans but highly virulent for mice. The cellular mechanisms of *F. novicida* infections are similar to those of fully virulent *F. tularensis*.

Despite recent advances, relatively little is known about the intracellular growth of *F. tularensis* and the bacterial factors that allow it to parasitize host cells and cause disease. *F. tularensis* uptake into macrophages is promoted by complement and to a lesser extent by types I and II class A scavenger receptors [11, 12, 13]. After entering macrophages *F. tularensis* initially

resides inside phagosomes. Shortly after entry *F. tularensis* escapes the phagosome and replicates in the cytoplasm of the host cell [14, 15]. *F. tularensis* induces autophagy vesicles at later time points of intracellular replication [16]. Although some *Francisella* genes are required for escaping the phagosome and intramacrophage growth, there has been no description of the molecular function of these virulence genes [15, 17, 18, 19].

Many of these virulence genes are encoded in the *Francisella* pathogenicity island (FPI), which contains 16 to 19 genes, depending on the subspecies. It is duplicated in all of the biotypes except *F. novicida* and *F. philamiragia*. The larger of the two presumed operons of the FPI is 17kbp long and contains a low G+C content of 26.6%, which is 6% lower than the 32.5% G+C content found in the rest of the *Francisella* genome [17]. While the majority of FPI genes are highly conserved among all strains, *F. tularensis* and *F. novicida* contain two genes, *anmK* and *pdpD,* that are not present in the FPIs of the other *F. tularensis* subspecies [17].

FPI-encoded proteins play essential roles in virulence and are co-ordinately regulated with other virulence factors. Disruption of many FPI genes including *igl*A [20, 21], *iglC* [18, 21-23], *iglD* [24], *pdpA* [17] and *pdpB* [25], leads to loss of intracellular growth or virulence of *F. tularensis* [26]. The *pdpD* gene is required for full virulence but is not necessary for intracellular growth [27]. *pdpC* in *F. tularensis* Live Vaccine Strain (LVS) is required for both intracellular growth and virulence, while *pdpC* from *F. novicida* is not required for intracellular growth [7, 26, 28]. However, very little is known about the precise roles of these FPI-encoded proteins *in vivo* and *in vitro* infection models. Micro-array data revealed all FPI genes are regulated by the global virulence regulator protein MglA [29]. MglA and another FPI regulator protein (SspA) interact with RNA polymerase in regulating numerous virulence genes including the FPI-encoded genes [30]. In addition, low-iron conditions in growth media up-regulate a number of

virulence-associated genes including FPI genes [31]. Bioinformatics analyses suggest several FPI genes encode proteins involved in a T6SS [6, 7, 20]. T6SS have been demonstrated in other pathogens such as *Vibrio cholerae* and *Pseudomonas aeruginosa* [32, 33, 34].

Pathogenicity islands in other bacterial pathogens often encode secretion systems, as well as secreted effector proteins. These effector proteins interact with host cells and can change host cell biology to favor survival and replication of pathogens. In LVS IglE, IglC, IglI, PdpE, PdpA, IglJ, IglF, and VgrG are secreted, and the secretion is dependent on DotU, VgrG, IglC, and IglG [6, 35]. IglE from type A is an outer membrane anchored lipoprotein required for phagosomal escape and intracellular growth [36]. PdpC, IglC, IglI, and IglG have roles in cytopathogenic effects such as mitochondrial damage, caspase-3 activation, and phosphatidylserine expression during infection of murine macrophages [37].

Based on PdpC's involvement in cytopathogenic effects of LVS and the inconsistencies of the role of PdpC during intracellular growth between different subspecies in recent studies, we examined the expression, localization, and role in virulence of PdpC in *F. novicida* [7, 28]. In addition the role of the gene downstream of PdpC, PdpE was also examined in virulence and its localization was examined in this study.

4.3 Materials and Methods

4.3.1 Bacterial Strains and Plasmids

F. novicida strains were grown aerobically at 37°C in tryptic soy broth supplemented with 0.1% cysteine (TSBC) or on trypticase soy agar supplemented with 0.1% cysteine (TSAC). The *F. novicida* JL0 strain is a derivative of the U112 prototype with a deletion in a sucrose hydrolase gene and is the parental strain of the Δ*pdpC* mutant [20]. The JL0 strain has a

phenotype in assays used in this study that is indistinguishable from the parental type strain U112, and was used as the wild type strains in experiments [20]. Erythromycin (Em; 30μg/ml), hygromycin (Hyg, 200μg/ml) or kanamycin (Kan, 15μg/ml) was added as selection markers. The pCR2.1-TOPO vector (Invitrogen) and pFNLTP6-GroE-GFP were used to clone PCR products [36]. Bacterial strains and plasmids used in this study are listed in Table 4.1.

4.3.2 Polymerase Chain Reaction (PCR) and Primer Design

Routine PCR reactions used for screening or analysis of mutants were carried out using Taq polymerase. For the creation of deletion mutants and chromosomal integration of the 3xFLAG epitope tag, overlap extension PCR was used to join two PCR amplicons. For these reactions or any amplification requiring proof-reading or highly processive enzyme reactions, Phusion DNA Polymerase (New England BioLabs®) was used in a reaction containing nuclease-free water, 1X Phusion HF buffer, 200μM dNTPs, 0.5μM each of the forward and reverse primers (Integrated DNA Technologies), 0.1ng of template DNA, and 1 unit of Phusion DNA Polymerase. PCR reactions were as follows: Initial denaturation at 98°C for 30s; 35 cycles of 98°C for 30s, 55°C for 30 s, 72°C for 2min 30s; final extension at 72°C for 10min. All primers were designed based on the *F. novicida* U112 genome sequence (GenBank accession no. NC 008601) and are listed in Table 4.2.

4.3.3 Subcellular Localization of PdpC

F. novicida expressing PdpC-FLAG was grown in 20ml trypticase soy broth-cysteine (TSBC), harvested by centrifugation, suspended in 1.0ml phosphate buffered saline (PBS) containing protease inhibitor (EMB Bioscience), and lysed by intermittent sonication and cooling

on ice 10 times for a total of 300s. Unbroken cells were removed by centrifugation twice at 22,000 x *G*, 4°C, 20min. Soluble and insoluble fractions were separated by ultracentrifugation as described previously [20]. Briefly, the soluble fraction was centrifuged for 1h at 100,000 x *G* (Beckman Optima TLX ultracentrifuge, TLA-100.3 rotor) to remove residual membrane proteins, and the insoluble pellet was washed once in ice-cold PBS. For preparation of outer-membrane fractions, Sarkosyl (Sigma Aldrich®) was added to cleared lysates to a final concentration of 1%, followed by ultracentrifugation for 1h at 100,000 x *G* at 4°C to pellet the outer membrane. Outer membranes were washed once in PBS to remove excess Sarkosyl. To normalize loading during SDS-PAGE and Western blots, protein content was determined by Bradford assay (Bio-Rad) and equal amounts of protein were analyzed for each sample.

4.3.4 Targeted Integration of FLAG Tag into the *F. novicida* Chromosome

Using the plasmid leading to the expression of C-terminal FLAG-tagged PdpC (pKH5) as the template, a 0.8kbp PCR product was amplified from the 3-prime end of *pdpC* including the FLAG tag. Next a 0.8kbp product immediately downstream of *pdpC* was amplified from *F. novicida* using a forward primer in which a portion of the 5-prime end was complementary to part of the FLAG tag. The two amplicons were purified using Wizard DNA purification system (Promega Madison, WI, USA). Purified products were diluted 100x in water and overlap extension PCR was subsequently employed to fuse the two amplicons together. The resulting PCR product was purified and integrated into the chromosome via co-transformation [38]. The pMP527 isolated from *E. coli* was transformed and purified from *F. novicida* prior to use in order to increase transformation efficiency. 870ng of pMP527 was mixed with 8 µg of PCR product and then transformed into *F. novicida*. Transformants were picked onto new plates and

successful integration screened for by colony PCR using forward and reverse primers within *pdpC* and the FLAG tag respectively. FLAG tag integrates were screened for the loss of pMP527 by growing isolates in antibiotic-free media and replica plating the resulting colonies onto TSAC plates with and without kanamycin. A Western blot of whole cell lysates from kanamycin sensitive integrates was probed with monoclonal M2 mouse anti-FLAG® antibodies (1/5000) (Sigma Aldrich®) to confirm that a reactive product of the predicted mass of PdpC was present.

4.3.5 Chemical Transformation of *F. novicida*

F. novicida strains were grown in fresh TSBC supplemented with 0.4% glucose until the exponential phase of growth. Cells were pelleted at 5,000 x *G* in a Beckman JA-20 rotor and suspended in *Francisella* transformation buffer (FTB) at room temperature [39]. Plasmid DNA PCR product or a ligation mixture, in a volume up to 100µl, was added to 200µl of suspended cells. The mixture was incubated at 37°C with shaking at 95rpm for 1h. One ml of TSBC supplemented with 0.4% glucose was added and the mixture was further incubated at 37°C for 4h with vigorous shaking. Transformants were selected on TSAC with antibiotics as needed for 24-48h.

4.3.6 SDS-PAGE and Western Blotting

Protein concentrations of subcellular fractions were determined by the BCA assay (Pierce) and 5µg/lane of protein was loaded. Samples of whole cell lysates were mixed with SDS sample buffer containing 62.5mM Tris (pH 6.8), 1% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol; and boiled for 10min prior to electrophoresis. SDS-PAGE was carried out using standard methods. Samples were electrophoresed through 8% SDS-PAGE

gels and transferred to Immobilon-P membrane (Millipore Billerica, MA USA). Membranes were blocked with 5% skim milk in (0.2M NaCl, 4.2mM KCl, 12.7mM Na2HPO4, 2.3mM KHPO4). Native PdpC was detected using rabbit polyclonal immune serum raised against a PdpC peptide (DDINVDRENRRELVAK) found at amino acids 168-183. After incubation overnight, blots were washed with PBS containing 0.1% Tween® 20 (Fisher BioReagents® Fair Lawn, NJ US) for 15min three times and subsequently probed with IRDye800DX-conjugated goat anti-rabbit Immunoglobulin G (Rockland Immunochemicals). Immunoblots were visualized using the LiCor Odyssey imaging system. The anti-PdpC polyclonal antibody, anti-IglB monoclonal antibodies, and source hybridomas were deposited with the BEI program of the American Type Culture Collection. Rabbit polyclonal anti-PdpC peptide antiserum was used to detect PdpC. Monoclonal anti-IglB peptide antiserum was used to detect IglB. The outer membrane protein, FopA, was detected using rabbit polyclonal anti-FopA as a control for approximate normalization for the other protein bands. Epitope tagged PdpC or PdpE was detected using the monoclonal M2 mouse anti-FLAG® antibodies (Sigma Alrich®) followed by anti-mouse HRP conjugate and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used as a substrate.

4.3.7 Mouse Infections

Six-to-eight week-old male specific-pathogen-free BALB/cByJ mice were purchased from the Jackson Laboratory. Animals were housed in sterile micro-isolator cages in barrier environment at the Center for Biologics Evaluation and Research. Mice were fed autoclaved food and water *ad libitum*. All experiments were performed under Institutional Animal Care and Use Committee guidelines (CBER ACUC # 93-03). Mice were given 0.1ml of appropriately

diluted bacteria intradermally at the base of the tail; actual doses of inoculated bacteria were simultaneously determined by plate count. The following numbers of mice used were: *F. novicida* U112: 12; *F. novicida* JLO: 10; Δ*pdpC*: 44; and *mglA* mutant: 12. All materials used in animals, including bacteria, were diluted in PBS (BioWhittaker) containing <0.01ng/ml endotoxin. The mean survival curves of mice infected with different strains of *F. novicida* were generated with Kaplan Meier survival curves.

4.3.8 Chicken Embryo Infections

F. novicida strains were grown to the late log phase (optical density at 600nm, 0.9 to 1.0) and diluted in PBS for injection. Inoculating doses were determined through colony-forming units (cfu) counts. Day old fertilized White Leghorn eggs were obtained from Charles River Labs (Wilmington, MA), and the eggs were incubated at 37°C with high humidity and mechanically tilted to a 45° angle every hour for seven days prior to infection and throughout the experiment. A lancet was used to puncture the eggshell at the air sac end of seven day-old fertilized eggs. With a tuberculin syringe, 100µl of inoculum was injected under the chorioallantoic membrane. After injection the shells were sealed with a drop of Elmer's® School Glue (Elmer's Products Inc. Columbus, OH). The following numbers of embryonated eggs used were: *F. novicida* U112, 58; Δ*pdpC*, 35; *F. novicida* Δ*pdpC* + FLAG-PdpC, 14; *F. novicida* Δ*pdpC* + PdpC-FLAG, 11; and PBS 28. The viability of the embryos was determined by illuminating the eggs from the bottom using a focused light. Embryos that had lost the integrity of the network of capillaries were scored as dead. Embryos that died within 24h of inoculation were assumed to have suffered lethal trauma during the inoculation and were removed from the experiment. All experiments were terminated by the time the embryos were 14 day-old. PBS was

also inoculated in a control group, and all PBS injected eggs survived the experiments. The mean survival times of chicken embryos infected with different strains of *F. novicida* were determined with Kaplan Meier survival curves. Differences in survival were determined using a Log rank statistic with $\alpha = 0.05/(k-1)$, and k = number of strains being compared.

4.3.9 Immuno-fluorescence Microscopy

J774 murine macrophage like cells were grown on coverslips for 16-24h before being infected with *F. novicida,* which was grown overnight at 37°C in TSB. Cells were infected for 1h at an MOI of 50, washed with PBS and incubated in DMEM containing 10% newborn calf serum. At the indicated times after infection, cells were fixed in 4% paraformaldehyde for 15min at room temperature. FLAG-tagged proteins or *Francisella* were detected with a 1h incubation of monoclonal M2 mouse anti-FLAG® antibodies (Sigma Aldrich®) and rabbit anti *Francisella* serum (Abcam), respectively. Antibodies were diluted (1/500) in PBS containing 0.5% BSA and 0.1% saponin to permeabilize host cells and not the bacteria [40]. Primary antibodies were detected using goat anti-mouse and goat anti-rabbit serum conjugated to Alexa Fluor® 488 and 594 (Life Technologies), respectively that were diluted (1/500) in PBS containing 0.5% BSA and 0.1% saponin. DNA was detected with DAPI for 10min after antibody staining. Coverslips were mounted using ® Gold Antifade reagent (Invitrogen™ MOLECULAR PROBES® Eugene, OR US) and examined using an Olympus TE81 inverted fluorescent microscope with spinning disc capabilities. Images were collected as Z-stacks and a projection image was generated using the Intelligent Imaging SlideBook™ software package. Using SlideBook™, masks were generated for macrophage, bacteria, and FLAG-tagged proteins. The percentage of bacteria associated with FLAG tag signal above background and the percentage of infected macrophages containing

detectable FLAG signal were determined for FPI proteins with a FLAG tag. Background fluorescent signal was determined by analysing images of cells infected with U112 not expressing a FLAG-tagged protein. Stacked three-dimensional photos of infected macrophages were collected. Three independent experiments were performed. The data were analysed by ANOVA with a left sided Dennett's test; comparing each protein's mean to the mean of the wild type not containing a FLAG expressing plasmid. Data were also analysed with Tukey multiple comparison test. For all statistical analyses $\alpha = 0.05$.

4.4 Results

4.4.1 PdpC Expression in *F. novicida*

To test if PdpC is expressed in *F. novicida,* an immunoreactive rabbit sera directed against a peptide sequence found in U112 PdpC was developed, that reacted with a 160kDa band of *F. novicida* lysates (Fig. 4.1A). Differential extraction showed U112 PdpC to be localized to the soluble, and to a lesser extent, inner membrane fraction of the bacterial cell (Fig. 4.1B).

4.4.2 The Roles of PdpC in Virulence of Mice

In order to determine if PdpC is required for virulence of *F. novicida* in a murine model, BALB/cByJ mice were infected with Δ*pdpC* mutant and wild type bacteria. Following an intradermal (i.d.) injection, the Δ*pdpC* mutant showed reduced virulence compared to its parental wild type strain (Fig. 4.2). While as few as 10^2 cfu of wild type U112 killed 50% of mice and 10^3 cfu of the parental JLO strain killed 70% of mice, 10^7 cfu of the Δ*pdpC* mutant killed only 11% of mice. The estimated i.d. LD_{50} in male BALB/cByJ mice for both *F. novicida* U112 and JLO was approximately 5×10^2, while the i.d. LD_{50} of Δ*pdpC* was greater than 10^7.

4.4.3 *pdpC* is Required for Virulence of *F. novicida* in Embryonated Chicken Eggs

To confirm the role of PdpC in a different *in vivo* model, fertilized chicken eggs were inoculated with 10^3 *F. novicida* and Δ*pdpC* mutants (Fig. 4.3). All embryonated eggs infected with *F. novicida* U112 died, and had a mean survival time of 2.72 days. As for eggs infected with Δ*pdpC*, 28 of 35 embryonated eggs died, and the mean survival time was 4.34 days.

The difference in the survival rate between the wild type and the Δ*pdpC* strain was significantly different ($p < 0.05$). Next the Δ*pdpC* mutant strains were complemented with a plasmids leading to the expression PdpC with FLAG tags at either the N- or C-terminus of the protein. Embryos infected with either complemented mutants died and showed a significantly different survival time from the Δ*pdpC* mutant ($p < 0.0001$) and similar survival rate to chicken embryos infected with the wild type bacteria ($p > 0.10$) (Fig. 4.3).

4.4.4 The Role of PdpE in Virulence

Since the *pdpE* gene lies downstream of the *pdpC* gene in an apparent operon, we were concerned that perturbations of the *pdpC* gene may affect the expression of *pdpE*, and perhaps the virulence phenotype. To test this possibility we studied the virulence of two *pdpE* mutants generated through transposon insertions. Comparison of the outcome of i.d. infections of BALB/cByJ male mice with Δ*pdpE*, similar to those performed to study the Δ*pdpC* mutant, showed *pdpE* mutants were as virulent as the wild type *F. novicida* strain (Table 4.3). All mice infected with the transposon insertion mutants of *pdpE* died.

The virulence of PdpE was also examined in embryonated chicken eggs. All infected eggs died when infected with U112 or *pdpE-1* mutant; the mean survival times were 2.7 days and 2.4 days, respectively (Fig. 4.4). The *pdpE-1* mutant is as virulent as the wild type *F. novicida* strain (Fig. 4.4). Therefore *pdpE* is not required for virulence in mice.

4.4.5 PdpC is Localized to the Host Cells During Infection

Using PdpC-FLAG expressed from a plasmid under the control of the GroEL promotor in *F novicida*, PdpC was detected as an extra-cellular protein within infected J774 macrophage-like cells (Fig. 4.5). Thirteen percent of bacteria were associated with signal for PdpC-FLAG at 30min post infection within infected J774 cells. At 4h 5% of bacteria were associated with PdpC-FLAG signal, while at 8 and 12h those percentages were 35% and 20% respectively. These percentages are significantly higher than the control bacteria not expressing FLAG-tagged protein at 30min, 8h, and 12h ($p < 0.05$) (Fig. 4.5B). At 4h post infection, PdpC-FLAG co-localized with bacterial cells and also in punctate structures in close proximity to, but not co-localizing with bacteria within infected cells (Fig. 4.5A and S.Fig. 4.1).

At all time points examined, 55-80% of infected macrophages contained PdpC-FLAG, a percentage significantly greater ($p < 0.01$) than the 7% or less of macrophages infected with bacteria not expressing FLAG-tagged protein that showed detectable unspecific background signal (Fig. 4.5C).

4.4.6 PdpC Localization During Infection is Dependent on T6SS

To determine if PdpC localization is dependent on the FPI-encoded T6SS the PdpC-FLAG expressing plasmid was transformed into the Δ*pdpB F. novicida* mutant, and the

localization of PdpC-FLAG was determined and compared to the localization of PdpC-FLAG in wild type *F. novicida*. Less FLAG signal was detected by immuno-fluorescence microscopy of cells infected with the Δ*pdpB* strain at 1, 4, and 12h post-infection; the difference between the two backgrounds were significant at 4 and 12h post-infection ($p < 0.05$) (Fig. 4.6A). The Δ*pdpB* mutant expressed PdpC-FLAG at levels similar to wild type *F. novicida,* and thus the difference in the extracellular localization was not due to a difference in expression level (Fig. 4.6B).

4.4.7 Localization of PdpC-FLAG to Host Cells Does Not Depend on Overexpression of PdpC-FLAG

The native *pdpC* in the *F. novicida* chromosome was replaced with an epitope 3xFLAG-tagged version of *pdpC*, *F. novicida* PdpC-FLAG to determine localization of PdpC at physiological expression levels. PdpC-FLAG co-localized with bacterial cells and was also detected in punctate structures in close proximity to, but not co-localizing with bacteria in infected cells (Fig. 4.7 and S.Fig. 4.2).

The native *pdpC* in the Δ*pdpB* mutant's chromosome was replaced with an epitope 3xFLAG-tagged version of *pdpC*, Δ*pdpB* PdpC-FLAG. The Δ*pdpB* mutant had less bacteria associated with PdpC-FLAG at 1, 4, and 12h post infection compared to the wild type bacteria *F. novicida* PdpC-FLAG expressing PdpC-FLAG; this difference between the two strains was significant at 12h ($p < 0.05$) (Fig. 4.7D).

4.4.8 Temporal Regulation of PdpC Secretion

The localization of PdpC-FLAG expressed from the endogenous promoter in the chromosome within J774 cells was observed over time (Fig. 4.8). PdpC signal was detected

above background in some infected cells post-infection, and steadily increased in signal intensity and frequency of infected cells (Fig. 4.8). By 13h a strong signal was observed in the majority of cells, and signal strength was maintained through 21h post-infection (Fig. 4.8). At 17 and 21h post infection, PdpC-FLAG had significantly more FLAG signal detected compared to U112 ($p > 0.0006$) (Fig. 4.8B)

4.4.9 PdpE is Secreted in a T6SS Dependent Manner

To test if PdpE was also detectable in infected host cells, *F. novicida* U112 wild type and Δ*pdpB* mutants were transformed with a plasmid, leading to the expression of a carboxyl triple FLAG-tagged form of PdpE, PdpE-FLAG. These bacteria were used to infect J774 cells, and the percentage of bacteria associated with PdpE-FLAG was determined (Fig. 4.9). Both the wild type and the Δ*pdpB* mutant expressed PdpE-FLAG at similar levels (Fig. 4.9A). At 4h post-infection of J774 cells with U112 expressing PdpE-FLAG, yielded clear staining for PdpE-FLAG associated with over a quarter of bacteria, while only 5% of Δ*pdpB* bacteria were associated with PdpE-FLAG (Fig. 4.9B). Immuno-fluorescence microscopy revealed PdpE-FLAG co-localized with bacteria in a unique studded configuration (Fig. 4.9C).

4.5 Discussion

Macrophages play a central role in defence mechanisms of mammalian hosts against invading pathogens. This cell type evolved to take up particulate matter through phagocytosis. The resulting phagosome travels along specific pathways fusing with other vesicles to finally deliver its content to the lysosome; a vesicular compartment with a low pH that contains proteolytic enzymes, which degrade proteins and lead to the killing of most bacteria. Intracellular

bacteria have evolved multiple mechanisms to evade this killing by macrophages, and can even exploit this cell type as a niche for their replication, thus avoiding detection by the adaptive immune system.

Secretion systems and their secreted effectors frequently mediate the interactions with host defences, such as survival of intracellular bacteria within host cells. Well-studied examples for such interactions are the type III secretion systems of *Salmonella* and *Shigella* and the type IV secretion system of *Legionella* [1, 2]. A T6SS has been described in *Vibrio cholerae*, *Pseudomonas aeruginosa*, and other pathogenic Gram-negative microorganisms [32, 33, 34]. In some of these pathogens a clear role in pathogenesis has been established, but in many more systems the role of these protein secretion systems awaits characterization. The *Francisella* pathogenicity island encodes one such T6SS [6]. Proteins encoded by the FPI are important in virulence and intramacrophage growth of *Francisella,* suggesting the T6SS plays a crucial role in the high virulence of this potential bioterrorism agent [7, 28].

In this paper the role of PdpC as a potentially secreted effector protein in *Francisella* pathogenesis has been examined. PdpC is expressed in LVS, while expression from U112 has not been confirmed to date [28]. PdpC-FLAG is mostly soluble with a small fraction also localized to the inner membrane fraction of the bacterium (Fig. 4.1B). Having mostly soluble properties suggests PdpC is soluble and could be localized as a bacterial cytosolic protein or a candidate protein for secretion beyond the bacterial membranes. In contrast, LVS PdpC is found predominantly as an inner membrane protein [28].

The Δ*pdpC* mutant is attenuated in mice and embryonated chicken eggs (Fig. 4.2 and Fig. 4.3). Plasmid expression of PdpC-FLAG in the Δ*pdpC* mutant restores the virulent phenotype to that of wild type in the embryonated chicken egg model (Fig. 4.3). The role in virulence of PdpC

in U112 is not dependent on intramacrophage growth [7]. In contrast LVS PdpC is required for intramacrophage growth and the LVS *pdpC* mutant significantly down-regulates IglB and IglD and moderately down-regulates IglC and IglA [28]. This down regulation of other virulence genes could explain the role of *pdpC* in intracellular growth of LVS.

The reduction of virulence observed with the deletion mutant could also be explained from polar effects on *pdpE,* the downstream and last gene in the operon. In all assays the *pdpE* mutant bacteria behaved similarly to wild type and did not show the virulence defects observed for Δ*pdpC* mutants in our studies (Table 4.3 and Fig. 4.4). These data are in concordance with results described for LVS PdpE [28]. These data show virulence phenotype of *pdpC* deletion is not due to polar effect on the expression of downstream genes.

This disconnect between virulence and intramacrophage growth phenotypes of *F. novicida* is unusual for FPI-encoded proteins and has so far only been described for *pdpD* [27], a gene also required for full virulence in animal models but dispensable for growth within macrophages. Most other FPI-encoded genes are required for both virulence and intramacrophage growth. This disconnect makes it unlikely that PdpC is a required functional component of the proposed T6SS, as a disruption of the secretion apparatus would render secreted effector proteins non-functional during infection.

Other bacterial pathogens encode their secretion system within plasmids and pathogenicity islands [41]. In addition to the structural components of secretion systems, these genetic elements often also encode secreted effector proteins [41]. This is not a surprising finding; it is unlikely that expression of a secretion system gives an evolutionary advantage to a bacterium if no secreted effector proteins have been acquired at the same time. To assess if PdpC is transported into the host cell as a potential effector protein during infection, epitope tagged

forms of PdpC were expressed in *F. novicida.* PdpC is detectable within infected host cells by immuno fluorescent microscopy (Fig. 4.5). Both plasmid and chromosomally expressed PdpC-FLAG are distributed in a punctate pattern in proximity to the bacteria but not always directly co-localized with *Francisella* (Fig. 4.5, Fig. 4.7, S.Fig. 4.1, and S.Fig. 4.2). Therefore, it is suggested that PdpC could target specific host structures and disrupt the host cell functions. The identity of these host structures are currently unknown, and their identification in future studies will greatly help in understanding PdpC's molecular function during the infection process. However, LVS PdpC induces mitochondrial superoxide production, mitochondrial damage, capase-3 activation, nucleosome formation, and phosphatidylserine expression [37]. While these results suggest PdpC is secreted during the infection of host cells, it is important to note that this interpretation is based on experiments with epitope-tagged proteins. This modification could alter secretion of proteins by the bacterium. During infection of host cells, PdpC is localized at a distance from bacteria; indicating PdpC is a secreted protein (Fig. 5.5, Fig. 4.7, S. Fig. 4.1, and S.Fig. 4.2). The reduction of FLAG signal observed in the Δ*pdpB* strain suggests PdpC is secreted by the proposed T6SS. LVS PdpC is localized to the bacterial inner membrane when assessed by broth-grown bacterial cell lysates, however, this mutant causes a down-regulation of *iglABCD,* which may affect the localization of PdpC, however, it is difficult to compare localization when performed under experimentally different conditions [28]. These differences may also be differences between the two subspecies and this difference could also explain the differences in results on the role of PdpC in intracellular growth between LVS and *F. novicida*.

The roles of PdpE in intramacrophage growth and virulence are consistent this study and previous studies in LVS (Fig. 4.4) [35]. In LVS PdpE is a secreted protein encoded in the FPI [35]. We therefore tested if PdpE is also secreted in *F. novicida*. PdpE co-localized with bacteria

in cells infected with *F. novicida* expressing an epitope-tagged form of the protein (Fig. 4.9C). The localization of PdpE is observed as unique studs co-localized around the bacterium. In cells infected with the Δ*pdpB* strain expressing the epitope tagged PdpE-FLAG, significantly less signal is observed despite similar expression levels of the protein in the Δ*pdpB* strain (Fig. 4.4). These reults suggest that protein localization is dependent on a functional FPI-encoded T6SS.

4.6 Conclusions

Data obtained in this study suggest PdpC is a secreted effector protein of *Francisella* that influences the virulence of this important potential public health threat. PdpC is required for full virulence in both animal models tested. PdpE on the other hand is not required for virulence. Immuno-fluorescence microscopy of both PdpC and PdpE reveals their locations as extracellular with respect to the bacterium in infected host cells. The localization of PdpC and PdpE is dependent on PdpB, suggesting that the FPI-encoded T6SS in required for the secretion of these two FPI-encoded proteins.

4.7 Authors' Contributions

RFH constructed epitope tagged plasmids, EBN, RFH and KH performed microscopy experiments and chicken embryo infections. FN, KLE, and KH designed the study. RFH, FN and KH wrote the manuscript. KKMC performed Western blots. EBN constructed most mutant strains. AYC performed mouse infection experiments.

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4.10 Chapter 4 Figure Legends

4.10.1 Figure 4.1 Expression of PdpC

Identification of PdpC through immuno blotting in whole cell bacterial lysates of wild type *F. novicida* and Δ*pdpC* mutant (A). Immuno blotting of *F. novicida* expressing PdpC-FLAG lysates in different cellular fractions: soluble fraction (S), membrane fraction (M), inner

membrane / sarkosyl soluble (IM), and outer membrane / sarkosyl insoluble (OM) (B). The amount of proteins applied to SDS-PAGE was normalized to 5 mg for each lane.

4.10.2 Figure 4.2 Survival of Mice Infected Intradermally with *F. novicida* and Δ*pdpC* Mutant

Adult male BALB/cByJ mice were infected intradermally (i.d.) with indicated strains and doses. Infection doses were confirmed by retrospective plate count. Results shown are combined from two independent experiments.

4.10.3 Figure 4.3 Survival of Embryos with Δ*pdpC* Mutants of *F. novicida*

F. novicida Δ*pdpC* mutant, *F. novicida* Δ*pdpC* mutant complemented by plasmid borne amino FLAG-PdpC or carboxyl PdpC-FLAG, and wild type *F. novicida* strain were injected into chicken embryos. All embryonated eggs were infected with indicated strains with an inoculum of 10^3. The viability of the embryos was observed for seven days.

4.10.4 Figure 4.4 Phenotype of *pdpE* Mutant *Francisella novicida*

Virulence of a *pdpE-1* insertion mutant was assessed in the chicken embryo model. Embryonated chicken eggs were infected with 10^3 cfu of indicated strains. Infection doses were confirmed by determining the colony forming units in the inoculum via retrospective plate count. Differences between the survival rates of embryonated eggs infected with U112 or *pdpE-1* insertion mutant are not significant ($p > 0.5$).

4.10.5 Figure 4.5 Detection of FLAG-tagged PdpC in Infected Host Cells

Macrophages were infected for 1h with *F. novicida* wild type (A top) and *F. novicida* expressing plasmid PdpC-FLAG (A middle and bottom). The left column represents FLAG and the middle column represents *F. novicida,* and the right column represents a merged image of the FLAG (green) and bacteria (red). Arrows indicate FLAG signal in infected cells not associated with bacterial staining (A bottom). The percent of bacteria co-localized with FLAG-tagged protein was determined at the indicated time points (B). The percent of infected macrophages with FLAG-tagged proteins (C). The graphs show the means and standard deviations from three independent experiments. Statistical significance is shown with an asterisk ($p < 0.05$). A portion of Figure 5A is shown as a three-dimensional reconstruction in supplemental files S.Fig. 4.1.

4.10.6 Figure 4.6 PdpC-FLAG Localization is Dependent on T6SS

The per cent of bacteria associated with plasmid PdpC-FLAG expressed in bacteria with different gen etic background, U112 and Δ*pdpB* (A). The graphs represent the mean and standard deviation from three independent experiments. Statistical significance is shown with an asterisk ($p < 0.05$). Expression of PdpC-FLAG from plasmid in whole cell lysates of wild type and Δ*pdpB* strain of U112 (B).

4.10.7 Figure 4.7 PdpC-FLAG Expressed From Bacterial Chromosome

J774 cells infected with U112 for 10h (A top row) or U112 expressing PdpC-FLAG from the bacterial chromosomal (A bottom row, B, and C). Arrows indicate FLAG signal in infected cells not associated with bacterial staining. The larger image in (C) representations the two-dimensional image with x and y-axes. The two smaller images are positioned in respect to the

larger image sharing either an x or y axes of the larger image with an additional z axes, allowing for a three dimensional view of the infected macrophage like cell. The percentage of bacteria associated with PdpC-FLAG from different backgrounds, U112 and Δ*pdpB* strains expressing chromosomal PdpC-FLAG during infection of cells was determined (D). The graph represents mean and standard deviation of three independent experiments. Statistical significance is shown with an * ($p < 0.05$). A portion of Figure 7C is available in supplemental material as a movie (S.Fig. 4.2).

4.10.8 Figure 4.8 Time Course of *pdpC* Detection in Infected Host Cells

J774 cells were infected with *F. novicida* carrying the *pdpC*-FLAG in the chromosome and fixed at indicated time points after infection. The top row shows cells infected with U112 not expressing epitope tagged protein 9h after infection. The per cent of bacteria associated with PdpC-FLAG was calculated, and the graph represents two independent experiments and error bars represent the standard deviation (B). Statistical significance based on Tukey multiple comparisons test is shown with an * ($p < 0.0006$).

4.10.9 Figure 4.9 PdpE-FLAG Expressed in Wild Type or *pdpB* Mutant Background

Expression of PdpE-FLAG from plasmid in whole cell lysates of wild type and Δ*pdpB* strain of U112 (A). J774 cells were infected with U112 and Δ*pdpB* expressing PdpE-FLAG. Automated analysis of the per cent bacterial cells associated with FLAG signal in wild type and Δ*pdpB* backgrounds at 8h post infection (B). Three-dimensional reconstruction of the infected

cell with perspective views of the Z-stack from the left and the back the image with U112 expressing PdpE-FLAG (C).

4.10.10 S. Figure 4.1 Three-dimensional Reconstruction of PdpC-FLAG in Infected Cells

J774 macrophage cells were infected with U112 expressing PdpC-FLAG from a plasmid and were processed for immuno-fluorescence 1h after infection. Confocal images were processed to give a 3D representation of the infected cell. DAPI is shown in blue, anti *F. novicida* is shown in red and anti-FLAG is shown in green.

4.10.11 S. Figure 4.2 Three-dimensional Reconstruction of PdpC-FLAG in Infected Cells

J774 macrophage cells were infected with U112 expressing PdpC-FLAG from the bacterial chromosome and were processed for immuno-fluorescence 8h after infection. Confocal images were processed to give a 3D representation of the infected cell. DAPI is shown in blue, anti *F. novicida* is shown in red and anti-FLAG is shown in green.

Chapter 4 Tables

Table 4.1 Bacterial Strains and Plasmids

Strains	Relevant Characteristics	Source or Reference
E. coli DH5α	*sup*E44 *lac*U169 (Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Laboratory strain
pCR2.1	*E. coli* Cloning vector: Ap^R Km^R	Invitrogen
pWSK29	*E. coli* vector: Low-copy-number, Ap^R, *lacZ*α gene	[42]
pSMART	*E. coli* vectors: Low-copy-number, Ap^R, *lacZ*α gene	Happy Corp.
U112	*Francisella novicida* wild type strain	ATCC
JL0	U112 with deletion in FTN_1390	[20]
LVS	*F. holarctica* Live Vaccine Strain	ATCC
GB2	U112 with a transposon insertion mutation in *mglA* gene.	[43]
Δ*pdpC*	JL0 with deletion of the complete *pdpC* structural gene	This study
Δ*pdpB*	JL0 with deletion of the complete *pdpB* structural gene	[7]
pdpE-1	U112 Tn*5* insertion in *pdpE* at nucleotide 210	[44]
pdpE-2	U112 Tn*5* insertion in *pdpE* at nucleotide 382.	[44]
pMP633	Hygromycin resistant *Francisella* plasmid	[45]
pMP527	Kanamycin resistant *Francisella* plasmid	[45]
pKH5	U112 + *pdpC*-3xFLAG in pFNLTP6-GroE	Chapter 3
pKH16	U112 + *pdpE*-3xFLAG in pFNLTP6-GroE	Chapter 3

Table 4.2 Primers Used in Mutant Construction, FLAG Tag Integration Complementation, and Sequencing

Primers	Sequence 5' -> 3'
Markerless pdpC Deletion (Δ*pdpC*)	
Cupflank_F (Primary primer, Upstream flank of *pdpC*)	TAAGGGTAAGAGGAGATTTATATGAGTCAG
UFDel_R(Primary primer, Upstream flank of *pdpC)*	CATATTTTTGCATCCTTAAGCTAATGTACTT CCTTAATTTATC
DFDel_F (Primary primer, Downstream flank of *pdpC)*	GATAAATTAAGGAAGTACATTAGCTTAAGG ATGCAAAAATATG
Cdwnflank_R (Primary primer, Downstream flank of *pdpC)*	TCTCAAGGAAGCTTGCCAGT
UF_C_xhoF (Secondary reaction primers)	CTCGAGCTTAGTCACTATGGATGC
L-big_C (Secondary reaction primers)	CTCGAGAGGTTAAGCACCGCAAGCTA
1318-F	GAGGATCCATGAAGACTATTTTGAAGATC
pdpM-pET-3	GTGCTCGAGTTATATTATAGTAATTTTCTTT TCATAATGAGG
pdpC 3xFLAG chromosomal integration primers	
1st *pdpC* back (pKH8 primers)	GCATCCTTAAGCTAGAGATCGTCATCCTTGT AATCG
1st *pdpE* forward (pKH8 primers)	GACGATCTCTAGCTTAAGGATGCAAAAATA TGAG
1st *pdpE* back	GAATTTGATTTCCAAAAGCC
2nd Forward	CTTAAAATTGCTCTTAATAATGCC
2nd Backwards	CTTGCTGATAGATTGTTAACTC

Table 4.3 Virulence of *F. novicida* Strains Following Intradermal Infection of BALB/c Mice

Strain	Infectious Dose*	Deaths per Total
U112	6.4×10^2	4/5
pdpE-1	2.5×10^2	5/5
pdpE-2	3.2×10^2	5/5
PBS	NA	0/7

Chapter 4 Figures

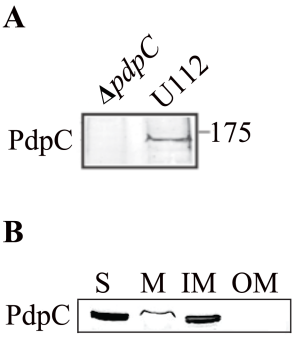

Figure 4.1 Expression of PdpC

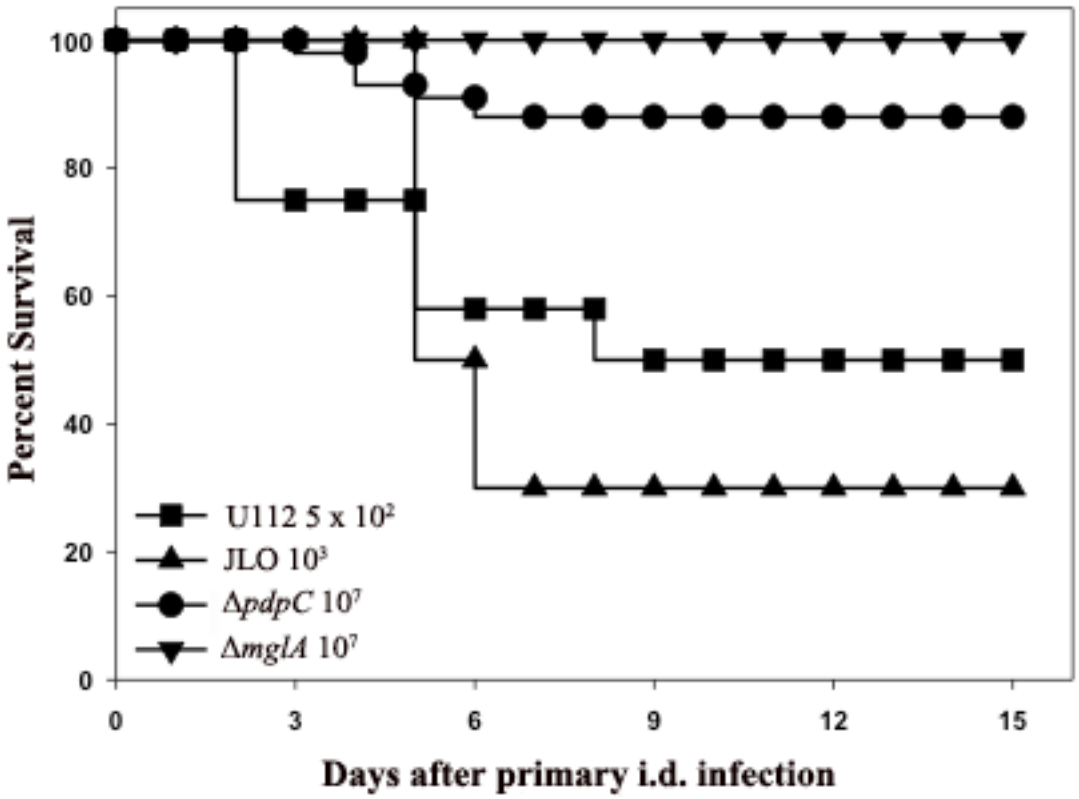

Figure 4.2 Survival of mice infected intradermally with *F. novicida* and Δ*pdpC* mutant

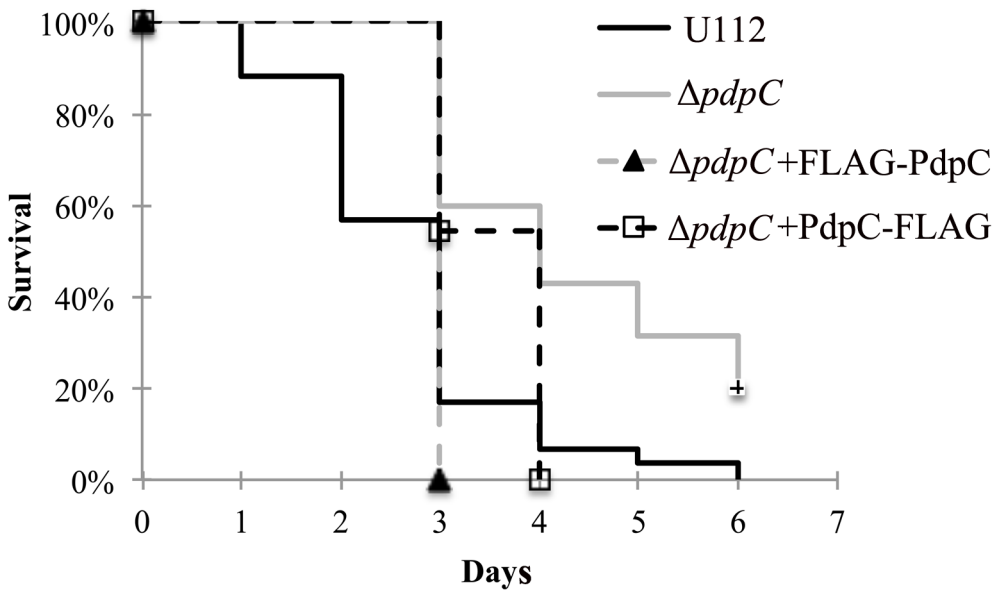

Figure 4.3 Survival of embryos with Δ*pdpC* mutants of *F. novicida*

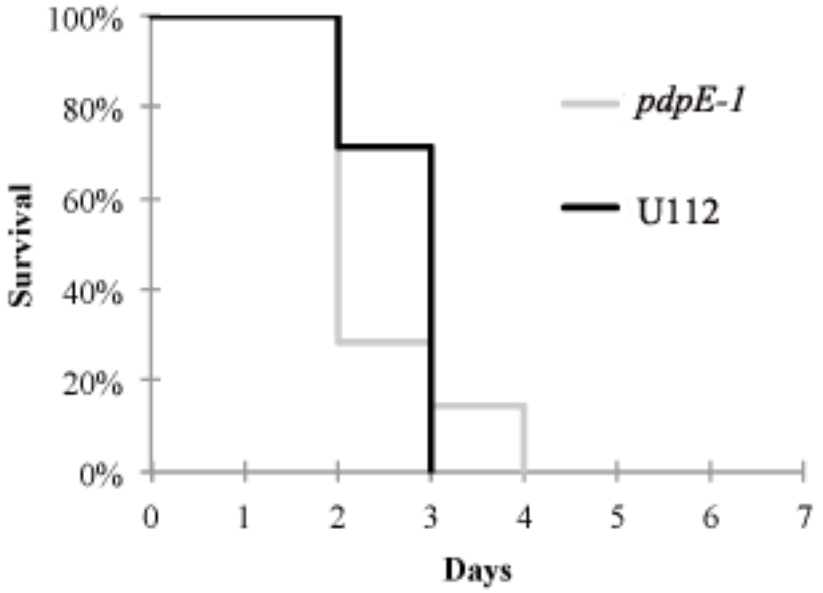

Figure 4.4 Phenotype of *pdpE* mutant *Francisella novicida*

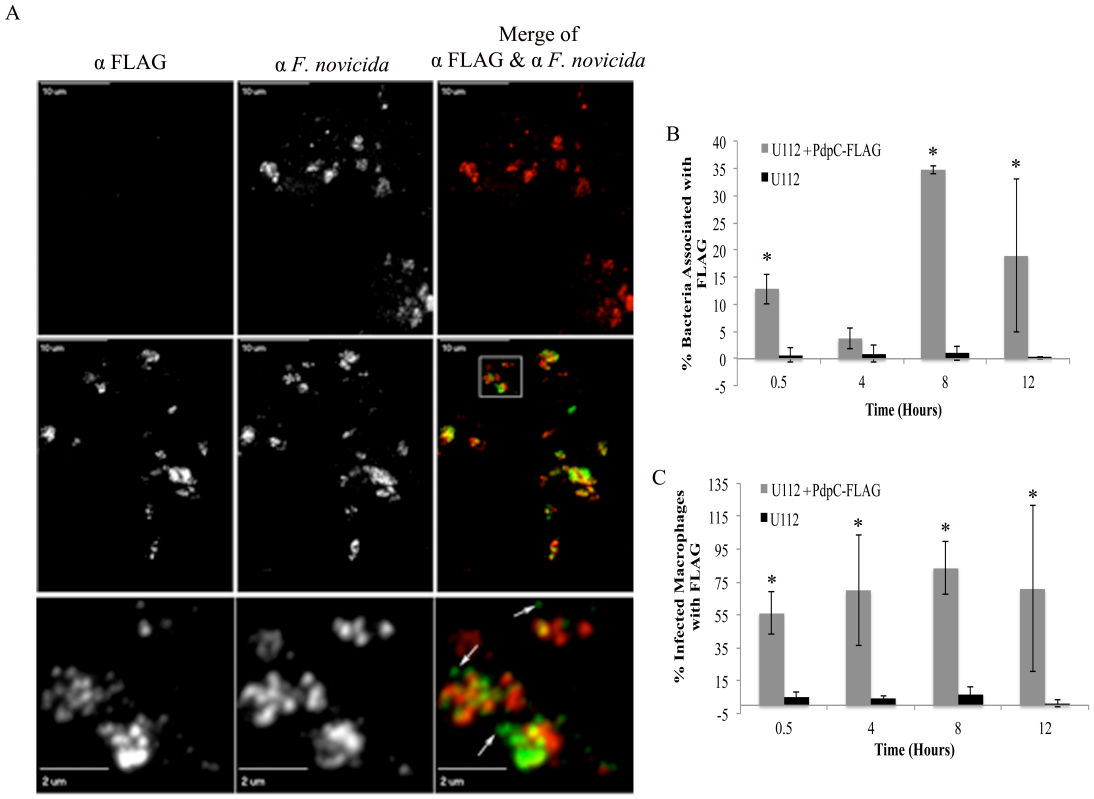

Figure 4.5 Detection of FLAG-tagged PdpC in infected host cells

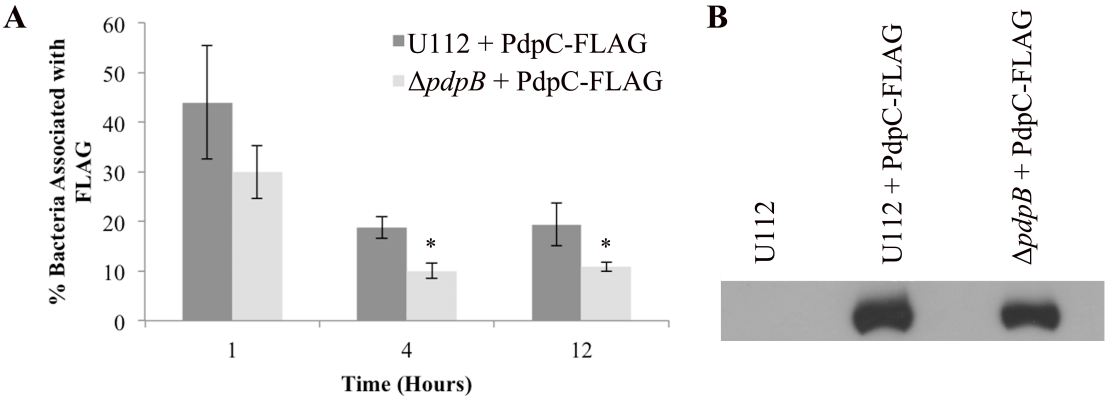

Figure 4.6 PdpC-FLAG localization is dependent on T6SS

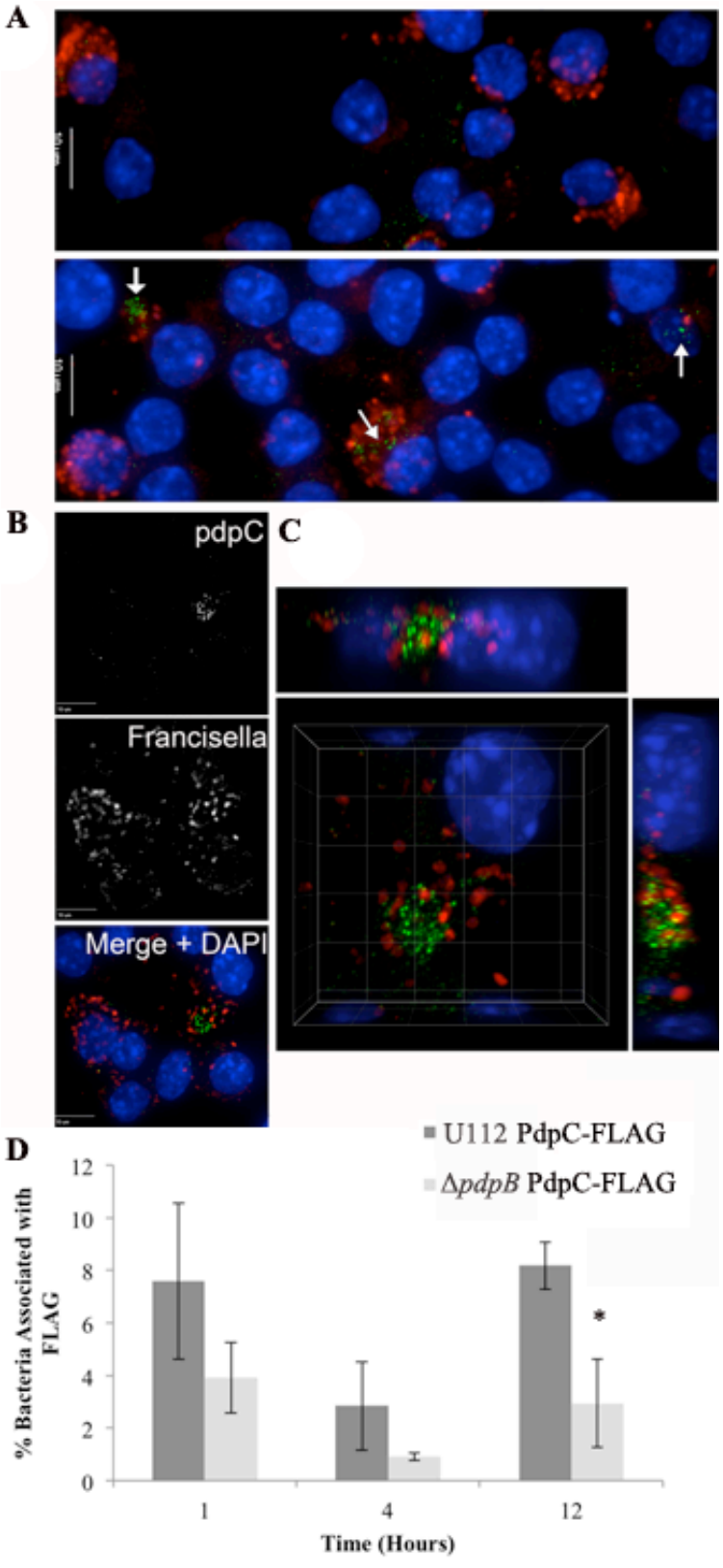

Figure 4.7 PdpC-FLAG expressed from bacterial chromosome

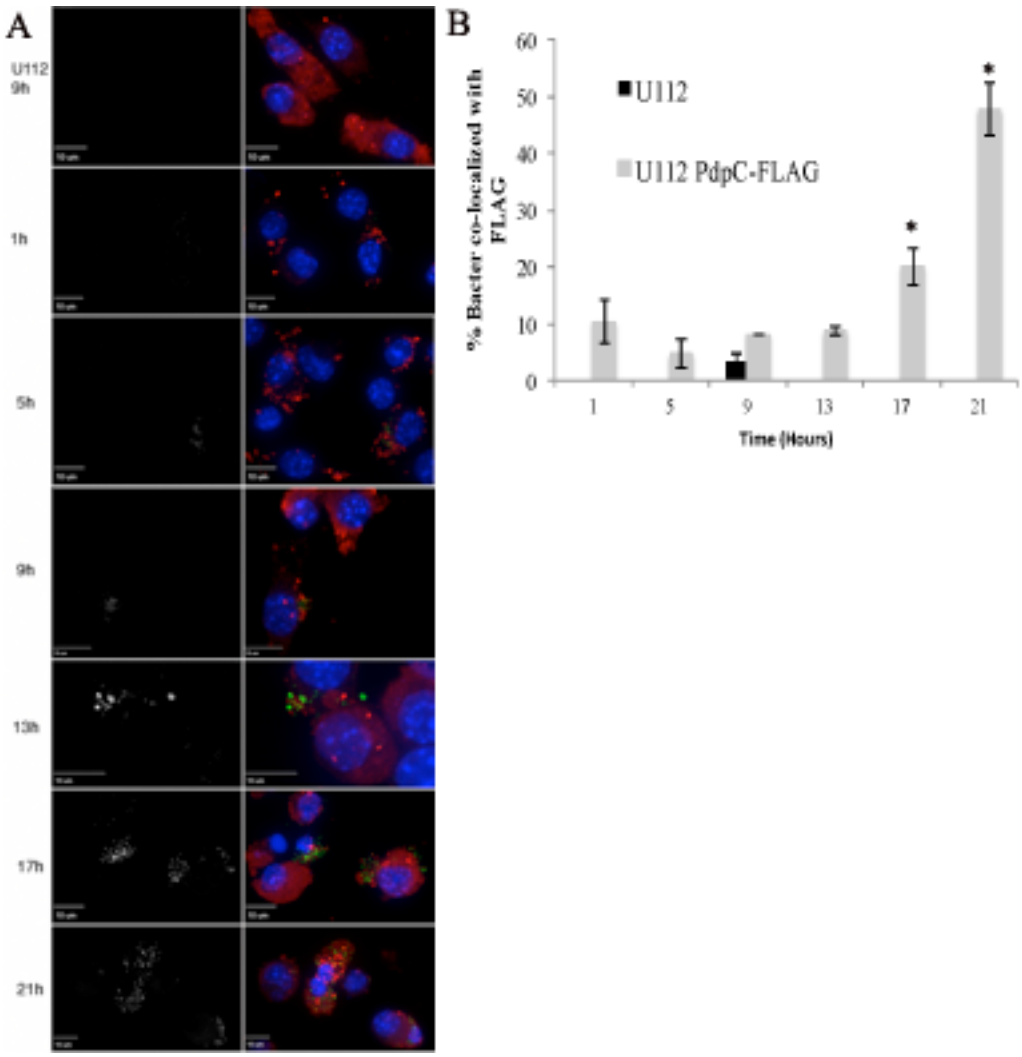

Figure 4.8 Time course of *pdpC* detection in infected host cells

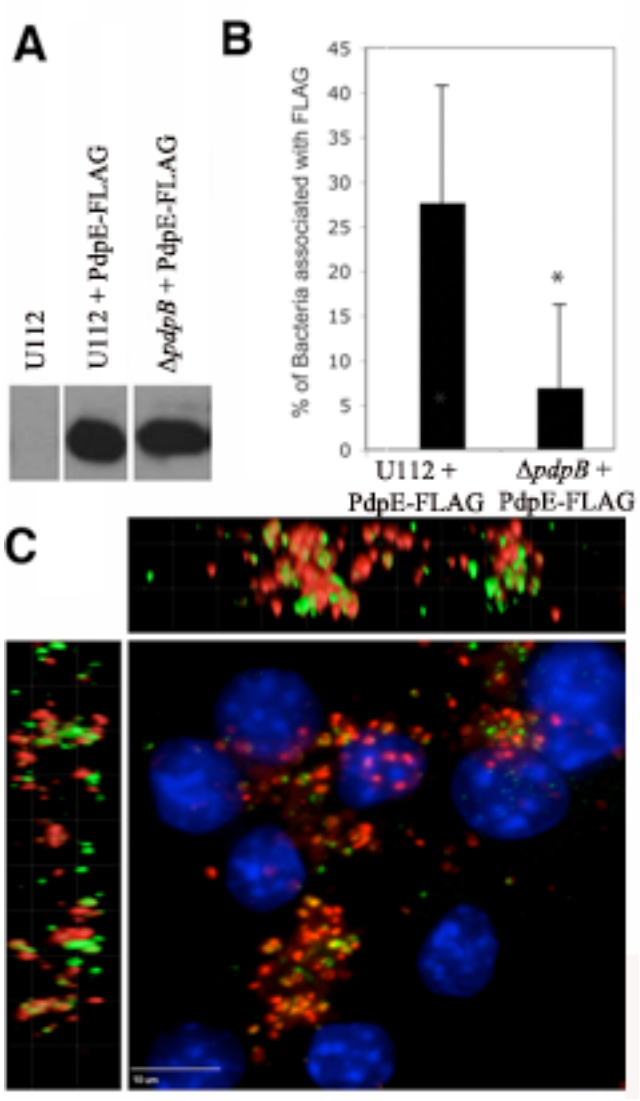

Figure 4.9 PdpE-FLAG expressed in wild type and *pdpB* mutant

Chapter 5: The Secretome within the FPI: Conclusions and Future Studies

Pathogenic bacteria evolved distinct interactions with their hosts that allow them to elude host defenses [1]. Horizontal gene transfer can drive this pathogen-host evolution through the acquisition of genes that encode for antibiotic resistance, pathogenicity islands (PI), virulence factors, and secretion systems [1, 2]. It is common for pathogenicity islands in bacteria to encode a secretome, including a secretion system, the proteins that are secreted, and chaperones of the secretion system [2].

Francisella tularensis is a facultative intracellular pathogen [3]. This organism avoids destruction and can replicate within macrophages [4]. The intracellular life cycle of this organism is unique and complex. *Francisella* contains many genes within its genome and specifically the *Francisella* pathogenicity island (FPI) that have not been previously characterized in other organisms [3]. Molecular mechanisms involved in survival and replication that are employed by *F. tularensis* remain poorly characterized. The FPI in *Francisella* is associated with virulence, intracellular growth, and a type VI secretion system (T6SS) [5]. It is important to identify the specific function of FPI proteins to understand *Francisella* pathogenesis. Based on the fact that *Francisella* has a pathogenicity island that encodes a secretion system, I tested the hypothesis that the FPI encodes a secretome, including the secretion system and the proteins that are secreted.

Available genetic tools to study the molecular mechanisms employed by *Francisella* within host cells often have significant limitations. Plasmids that have been characterized for use in *Francisella* species contain large GFP cassettes or reporter fusion tags that express non-functional proteins, making it impossible to complement mutations within genes required for intracellular growth [6, 7, 8]. In this project, genetic tools were designed to track proteins,

identify protein localization, and further understand the role and importance of these FPI proteins during infection of host cells. Through modification of the *Francisella* expression plasmid GroE-GFP- pFNLTP6, the *Francisella* expression plasmids were generated [6]. These plasmids express N-terminus and C-terminus FLAG-tagged FPI proteins at their predictive molecular weight. Through complementation of FPI gene mutants with defects in intracellular growth with the respective *Francisella* expression plasmids, all intracellular growth phenotypes were restored. This restoration of the wild type ability to replicate intracellularly supports that these proteins are biologically functional with either a C-terminus or N-terminus epitope FLAG tag.

Many of the FPI-encoded proteins are part of a T6SS, which led to the hypothesis that some of the FPI-encoded proteins may be directed for secretion by that secretion system, such as effector proteins or chaperones. Although some FPI genes are similar to those encoding T6SS in other organisms, there are several FPI genes that have no homology with genes that encode for T6SS [9, 10]. In this study *F. novicida* was used as a model for *F. tularensis* to identify the secreted FPI proteins by determining the intracellular localization of FPI-encoded proteins during infection of macrophage-like cells.

Microscopy of the FPI FLAG-tagged proteins revealed that several of these proteins are located within the outer-membrane or are secreted beyond the outer-membrane. Using the *Francisella* expression plasmids in *F. novicida* several proteins (PdpA, IglE, VgrG, IglF, IglG, IglH, DotU, IglI, IglJ, PdpC, PdpE, IglD, IglC, IglB, and IglA) were localized within macrophage-like cells on the outside of bacterial cell membranes. Some of the proteins were not detected in the cytoplasm of host cells; PdpB, PdpD, and Anmk were similar to the no plasmid expressing FLAG control, and were not secreted outside of the bacteria. This is consistent with previous studies showing these proteins are not secreted [9, 11].

The current model for the T6SS in *Francisella* includes inner-membrane anchor proteins of the transmembrane protein PdpB, which spans from the cytoplasm through the inner-membrane with parts also extending into the periplasmic space [11] (Fig. 5.1). The lack of PdpB detection supports the appropriateness of the employed techniques to only to detect FLAG epitope tagged protein outside the bacteria [12]. DotU is an inner-membrane component of the secretion system of *Francisella* and all T6SS's, and DotU interacts with IcmF, PdpB's homologue [11, 13]. Solubility properties suggest DotU is predominantly localized to the inner-membrane and periplasmic space and stabilizes PdpB and the secretion system [11, 13]. The localization of DotU within host cells has not been visualized before; it is intriguing that in this study, microscopy detected DotU as extracellular.

The inner-tube of the T6SS of *Francisella* is a polymer of IglC; which lies within the IglA and IglB polymer spanning through both the inner-membrane and the outer-membrane of *Francisella*, therefore they are exposed extracellularly and not necessarily secreted. When the IglA-IglB polymer contracts it drives the IglC polymer through the host cell membrane [11]. This contraction of IglA and IglB, could temporarily expose components of the secretion system (IglA, IglB, IglC, IglE, IglG, IglH, IglI, DotU, and possibly others) to extracellular staining (Fig. 5.1). VgrG and PdpE are located on the point of the secretion channel-forming tube and would therefore be expected to be detected outside of bacteria within host cells as shown in this study (Fig. 3.5 and Fig. 3.6) [11]. IglE is a lipoprotein localized to the outer-membrane, thus it is also accessible to staining of antibodies that are extracellular with to respect to the bacterial cell [14]. PdpD was not detected extracellularly and maybe another structural component of the T6SS that is unique to *Francisella. anmK* may have a role in restructuring the peptidoglycan to accommodate the T6SS machinery [15].

The localization of IglA, IglB, IglC, IglE, IglF, IglG, IglH, IglI, IglJ, DotU, VgrG, PdpE, and PdpB supports that these proteins are components of the T6SS (Fig. 5.1). While IglD, PdpA and PdpC could be potentially secreted proteins. It is not clear whether these proteins are secreted, localized to the outer-membrane of *Francisella*, or temporarily localized to the outer-membrane as components of the secretion system during the transport of other secreted proteins. Another alternative explanation is that their detection in this study is due to over expression from the *Francisella* expression plasmids.

The FPI gene products that do not clearly contribute to secretion could be substrates of the secretion system, either effectors or secreted chaperones (Fig. 5.1). IglD, PdpA, and PdpC are soluble proteins that were detected extracellularly and might function as secreted effecters or chaperones. IglD is required for phagosomal escape and may also be an effector protein that is secreted [16].

To test if protein localization is dependent on the T6SS, I compared the localization observed in WT with the localization of FPI proteins in a *pdpB* mutant, a gene that is homologous to a T6SS structural inner-membrane protein [17, 18]. This has been demonstrated with IglC in previous studies [8, 9, 11]. The localization of IglC and PdpE FLAG-tagged proteins was significantly reduced when expressed in the *pdpB* mutant compared to expression in wild type.

To further investigate the possible function of a potentially secreted effector protein with no described function, I focused on the localization of PdpC within macrophage-like cells and its role in virulence within animal models. In LVS PdpC is required for both intracellular growth and virulence [19]. Since PdpC and PdpE were secreted and their localization was T6SS dependent, their roles in intracellular growth and virulence in *F. novicida* were examined. *pdpC*

is required for virulence in mice but not for growth within macrophages of this *Francisella* species. *pdpC* is also required for virulence in chicken embryos, and plasmid expression of PdpC-FLAG and FLAG-PdpC in the *pdpC* mutant restored the virulent phenotype to that of the wild type. This virulence associated with PdpC was not due to effects of the downstream gene *pdpE*, as PdpE was not required for intramacrophage growth or virulence in mice. Data obtained in these studies suggest the FPI encodes a secretome consisting of the components of the secretion system, chaperones, and secreted proteins like PpdC, which was required for virulence.

The results of these studies will be foundations for future research projects focusing on the FPI and its encoded proteins in *Francisella* intracellular growth and virulence for years to come. These future studies include identifying the targets of the secreted proteins and their chronology of secretion. Previous studies of LVS PdpC, suggest a role in cytopathogenicity; this would be interesting to investigate with *F. novicida* [19]. Both LVS and *F. novicida* PdpC is required for virulence, yet their roles with intracellular growth are different [19]. In addition to identifying and characterizing effectors of the FPI-encoded secretion system, future studies should be aimed at characterizing the structure and interaction of FPI secretion system apparatus components. The genetic tools described in this study can be used in future studies to further characterize FPI proteins; there are a wide-range of commercial products specifically for FLAG tags that provide many opportunities for studying FPI proteins.

5.1 References

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5.2 Chapter 5 Figure Legend

5.2.1 Figure 5.1 Model of *Francisella's* T6SS

This model of the T6SS is based on the previously published model where PdpB and DotU interact in the inner-membrane (IM), IglA and IglB interact as an outer-tube surrounding the inner-tube IglC, and VgrG and PdpE make the cap and point of the secretion system [11]. IglI and IlgE are localized to the outer-membrane (OM). IglFGHJ were localized to IM / periplasmic space. PdpD and Anmk remain inside the bacterial cytoplasm. PdpA, PdpC, and IglD are secreted. Green and blue proteins were localized extracellularly during immuno-fluorescence microscopy. The yellow, orange, red, and pink proteins remained intracellularly.

Chapter 5 Figures

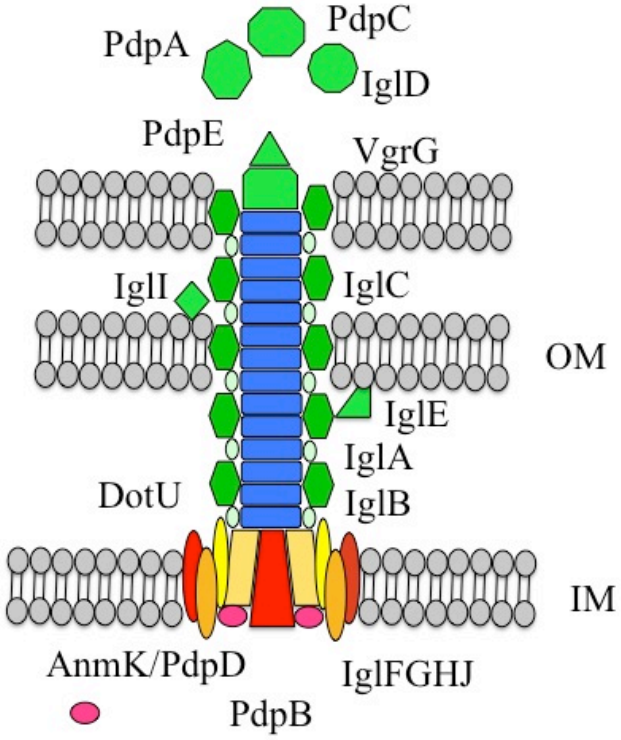

Figure 5.1 Model of *Francisella's* T6SS